REMARKS/ARGUMENTS

Claims 23, 24, 26, 27, 31, 41, 42 and 45-53 are pending in the application. Claims 23, 24, 26, 27, 31, 41, 42 and 45-53 are rejected. By the present amendment claims 24, 26, 27, 31, 41, 42, 45-50 and 53 are amended. Claims 23, 51 and 52 are cancelled. The amendments add no new matter.

Statement of the substance of the interview of November 18, 2003:

Applicant thanks Examiner Shahnan Shah, her Primary Examiner, Rodney Swartz, and USPTO SPRE Dr. Brian Stanton for the telephone interview of November 18, 2003, with Applicants' representatives Diane Dobrea (reg # 48,578) and Pamela Docherty (Reg # 40,591), when the status of the application and issues relating to enablement and the scope of the claims were discussed. During the interview, Applicant's representatives were advised that (1) the application would not be abandoned, and (2) Applicants' request for reconsideration of the finality of the rejection of the last Office action was deemed to be persuasive, and therefore, the finality of the rejection would be withdrawn by the Patent Office and Applicant would be provided with an opportunity to provide further arguments and amendments in response to the prior office action and consistent with the interview discussion.

Applicant has reviewed the Interview Summary form prepared by Examiners Shahnan Shah and Rodney Swartz, and Applicant concurs that the interview involved an in depth discussion of the scope of the claims and enablement thereof. Applicant concurs that the interview also involved discussion of references which support for the use of rabbits as an animal model for human Anthrax disease and metalloproteinase mutations to LF.

Applicant has amended the claims of the present application consistent with the suggestions of the Examiners and SPRE Stanton during the interview of November 12, 2003, and subsequent discussions with the Examiners. Specifically, Applicant has amended the claims regarding recitation of the description of the polynucleotides and their products that comprise the immunogenic compositions. As requested by the Examiners and SPRE Stanton, Applicant has also submitted as exhibits to this Response and Amendment which include the following:

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A Lethal Factor Active-Site Mutations Affect Catalytic Activity In Vitro S.E. Hammond and P.C. Hanna, Infection and Immunity 66: 2374-2378, 1998.

B Klimpel, K.R., Arora, N., and S.H. Leppla, 1994, Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. Mol. Microbiol. 13: 1093-1100.

C Zaucha GM, Pitt LM, Estep J, Ivins BE, Friedlander AM. Arch Pathol Lab Med. 1998 Nov;122(11):982-92.

References A and B, which were published prior to the filing of the present application, describe a variety of different LF mutations which alter or destroy metalloproteinase activity.

Reference C, which was published prior to the filing of the present application, has been used by the FDA as the basis for accepting the rabbit as a model for human Anthrax disease.

In view of the above-described amendments and supporting documents, reconsideration of claims 24, 26, 27, 31, 41, 42, 45-50 and 53 is respectfully requested. It is submitted that claims 24, 26, 27, 31, 41, 42, 45-50 and 53 are now in condition for allowance. Prompt notice of such allowance is respectfully requested.

Respectfully submitted,

Date: Dec 18, 2003

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INFECTION AND IMMUNITY, May 1998, p. 2374–2378
0019-9567/98/$04.00+0
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Vol. 66, No. 5

Lethal Factor Active-Site Mutations Affect Catalytic Activity In Vitro

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Received 26 June 1997/Returned for modification 11 August 1997/Accepted 31 January 1998

The lethal factor (LF) protein of *Bacillus anthracis* lethal toxin contains the thermolysin-like active-site and zinc-binding consensus motif HEXXH (K. R. Klimpel, N. Arora, and S. H. Leppla, Mol. Microbiol. 13:1093–1100, 1994). LF is hypothesized to act as a Zn^{2+} metalloprotease in the cytoplasm of macrophages, but no proteolytic activities have been previously shown on any target substrate. Here, synthetic peptides are hydrolyzed by LF in vitro. Mass spectroscopy and peptide sequencing of isolated cleavage products separated by reverse-phase high-pressure liquid chromatography indicate that LF seems to prefer proline-containing substrates. Substitution mutations within the consensus active-site residues completely abolish all in vitro catalytic functions, as does addition of 1,10-phenanthroline, EDTA, and certain amino acid hydroxamates, including the novel zinc metalloprotease inhibitor ZINCOV. In contrast, the protease inhibitors bestatin and lysine CMK, previously shown to block LF activity on macrophages, did not block LF activity in vitro. These data provide the first direct evidence that LF may act as an endopeptidase.

Lethal toxin (LeTx) is a vital virulence factor of *Bacillus anthracis* and has been postulated to act as a Zn^{2+} protease mediating the fatal symptoms observed during anthrax infections by hyperstimulation of host macrophage inflammatory pathways (5, 6, 8, 10). LeTx is an A-B toxin comprised of two distinct proteins. Protective antigen (PA; 735 residues, 82.6 kDa) serves as the B moiety, directing binding to cellular membrane receptors and translocation of its catalytic partners into the cytoplasm (5, 11). Lethal factor (LF; 776 residues, 90.2 kDa) acts as the A moiety (5, 11). Evidence presented by Klimpel et al. demonstrates that LF is a zinc-binding protein which contains the HEXXH motif in its carboxy-terminal (activity) region at residues 686 to 690 ($LF_{686-690}$) (10). They hypothesized that LF requires zinc for activity and perhaps functions as a Zn^{2+}-dependent protease, thus having functions similar to those of, and having an active-site motif in common with, the botulinum and tetanus neurotoxins, albeit with differing cell tropisms, target substrates, and disease sequellae (16). Here, we demonstrate LF-specific, Zn^{2+}-dependent cleavage of synthetic peptides in vitro. These data, as well as those from protease inhibitor profiles, metal ion substitution studies, and mutational analysis of residues within $LF_{686-690}$ that arrest activity, strongly support LF as demonstrating the activities of a Zn^{2+}-dependent neutral endoprotease.

Anthrax toxin purification. LF, PA, and mutants were purified either from *B. anthracis* Sterne or as recombinant proteins from *Escherichia coli* (6–8, 14). *B. anthracis* cultures were grown in defined RM toxin production medium (13). Culture supernatants were sterilized by passage through a 0.22-μm-pore-size filter (Millipore, Bedford, Mass.) and concentrated to 500 ml with the Minitan ultrafiltration system (Millipore). Ammonium sulfate was added to 75%, and the protein pellet was collected and suspended in 20 mM Tris-HCl (pH 8.0) and dialyzed extensively against the same buffer. Very efficient purification was performed by MonoQ anion-exchange fast-performance liquid chromatography (FPLC) (Pharmacia Biotech, Piscataway, N.J.). PA eluted at 130 to 140 mM NaCl, and LF eluted at 250 to 270 mM NaCl. The recombinant proteins expressed in pET15b are produced with an amino-terminal hexa-histidine tag, allowing purification by FPLC affinity chromatography on a HiTrap (Pharmacia Biotech) chelating column. Cultures of *E. coli* BLR(DE3)/pET15b-LF (or indicated LF mutants) were grown in Luria broth containing ampicillin (100 μg ml^{-1}) to an optical density at 600 nm of 0.7 to 1.0, and expression was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM) for 12 h at 18°C. Cell lysates were prepared by French press, cleared by centrifugation, and injected via FPLC (Pharmacia Biotech) onto a HiTrap column charged with Ni^{2+}. Recombinant histidine-tagged LF (wild type [wt] or mutant) eluted at approximately 100 mM imidazole. Eluted protein was further purified by gel filtration on a 320-ml Sephacryl-200 FPLC column. By these methods, PA and LF were each determined to be 95 to 99% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. PA and LF proteins were assayed for cytotoxicity activities by a standard ^{51}Cr release assay of a sensitive macrophage cell line (RAW 264.7 ATCC TIB-71) (11, 13). Mutant proteins LF^{E687C}, LF^{H686A}, LF^{H690A}, and $LF^{H686A+H690A}$ were obtained from Kurt Klimpel (10) as direct mutants of wt anthrax LF. LF^{E687D} was created as a recombinant mutant in *E. coli* BLR(DE3) by using the pET15b plasmid and purified as described above.

Protease assay conditions. Oligopeptides were obtained from Sigma. The reaction buffer used was 25 mM potassium phosphate (pH 7.0) containing 20 μM $ZnSO_4$ and 20 μM $CaCl_2$. Each reaction mixture contained as indicated from 250 μM to 1 mM substrate and 0.25 to 2.8 μM enzyme (23 to 250 μg/ml) in a final volume of 100 μl. After incubation at 37°C for 3 to 24 h (as indicated), the reactions were quenched with 1 μl of 10 M HCl and the mixtures were injected onto a C_{18} HP Hypersil-octyldecyl silane column (100 by 4.6 mm, 120-Å pore size, 5-μm particle size) or a C_8 Rainin Microsorb-MV column (250 by 4.6 mm, 300-Å pore size, 5-μm particle size) with a Hewlett-Packard 1050 high-performance liquid chromatography (HPLC) system. The aqueous phase used with the C_{18} column was 25 mM phosphate buffer (pH 7.5) with 2% (vol/

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vol) methanol (MeOH) and 2% (vol/vol) tetrahydrofuran, and the mobile phase was 100% MeOH. The aqueous phase used with the C_8 column was 0.1% (vol/vol) trifluoroacetic acid (TFA) in water, and the mobile phase was 0.1% (vol/vol) TFA in acetonitrile. Peptide peaks were detected by UV absorption at 215 and 274 nm. For further analysis by mass spectrometry or protein sequencing, peaks were collected as they eluted. For kinetic studies, reaction mixtures of 100 μM to 1 mM substrate with 250 nM LF were run at 25°C. Samples were injected into the HPLC at 3-h intervals. Velocities were then calculated by measuring the reduction of the starting substrate peak over time or by measuring the formation of product peaks over time. In inhibitor studies, enzyme and inhibitors were coincubated at room temperature for 1 h before addition of substrate. For studies with chelating inhibitors, there was no addition of exogenous metals. Otherwise, the conditions were the same as those described above. For pH studies, 250 ng of LF was added to 500 μM substrate in a buffer of 25 mM phosphate with pHs ranging from 5.8 to 8.2. Phosphate buffers were made from appropriate amounts of mono- and dibasic sodium phosphate. Reactions were analyzed after 4 h at 37°C as described above. Results indicated a pH optimum of approximately 6.75, with less than 5% activity at pH 5.5 (results not shown). Thus, the addition of HCl to reaction mixtures was determined to be the best way to quench reactions before HPLC analysis. To create apo-enzyme (no bound metals), purified LF was dialyzed against 10 mM EDTA–1 mM 1,10-phenanthroline for 24 h at 4°C. The proteins were then dialyzed against 20 mM Tris (pH 7.5) in ultrapure water (≥15 MΩ), for 24 h at 4°C, with four buffer changes.

Peptide fragment analysis. Peptides purified by reverse-phase HPLC (RP-HPLC) were analyzed by time-of-flight mass spectrometry. The mass spectrum was recorded by using nitrocellulose targets (9) in an Applied Biosystems Bio-Ion plasma-desorption instrument. The spectrum was accumulated for 10^6 fission events corresponding to approximately 10 min. Further details of the instrumentation and spectral analysis have been described elsewhere (19). Peptide analysis was kindly performed by the Duke Comprehensive Cancer Center Facility, directed by Jan J. Enghild.

Hydrolysis of synthetic peptides. To directly assess whether LF is capable of endopeptidase activity, synthetic peptides (or *p*-nitroanilide-derivatized peptides) as well as a variety of purified proteins were obtained as test substrates in an arbitrary manner. However, the lengths of the synthetic peptides ranged from 2 to 39 residues with efforts to vary both amino acid composition and primary sequence. The overwhelming majority of these substrates were not affected by LF in any discernible way, although they were cleaved by appropriate control proteases (e.g., trypsin, pronase, thermolysin) (data not shown). A complete list of substrates tested in this study is available upon request.

Sixteen oligopeptides 6 to 21 residues in length containing a large variety of amino acids were then obtained and assayed as LF substrates (Table 1). Results presented in Fig. 1 indicate LF cleavage of three peptides. Data illustrated in Fig. 1A show significant hydrolysis of peptide 1 (with ELYENKPRRPYIL hydrolyzed into ELYENKPRRP and YIL) after incubation with LF. The k_{cat}/K_m for this reaction was calculated to be 8.9 s^{-1} M^{-1}. Peptide 2, FGFLPIYRRPAS, was hydrolyzed by LF into the major products FGFLP and IYRRPAS, as well as minor products FGF and LPIYRRPAS (Fig. 1B). The k_{cat}/K_m for this reaction was calculated to be 29.4 s^{-1} M^{-1}. It is important to note that this value reflects the consumption of substrate by both major and minor reactions. Peptide 3, IARRHPYFL, was hydrolyzed by LF into the major products IARRHP and YFL and the minor products IARR and HPYFL (Fig. 1C). Kinetics were not determined for peptide 3; however, the cleavage of peptide 3 occurs faster than that of either peptide 1 or peptide 2 and could have a k_{cat}/K_m value greater than 50 s^{-1} M^{-1}. Unlike peptide 2, in which FGFLP is not split into FGF and LP, the peptide 3 major product IARRHP is hydrolyzed to IARR and HP. These three proteins have some sequence homology at their major cleavage sites, the most notable of which is proline at the new C terminus. The requirement for additional amino acids at key positions is clear from peptides that contain proline but show no signs of cleavage. Additionally, tyrosine is consistently present at or adjacent to the new N-terminal side of the cleavage site. Whether LF requires a tyrosine or simply a bulky uncharged residue is still under investigation. Cleavage of peptide 4 (YGGFLRRI) into YGGFLR and RI is similar to the secondary cleavage of peptide 3, that is, IARRHP to IARR and HP, and occurs at approximately the same rate. Cleavage of peptide 5 (DRVYI HPFHL) occurs extremely slowly into the three products DRV, YIHP, and FHL. No evidence of possible intermediates DRVYIHP and YIHPFDL could be found. Due to the very poor nature of this substrate, drawing similarities between this reaction and the others, while possible, may lead to incorrect assumptions.

The calculated reaction rates are considerably lower than what is normally seen in general metalloproteases such as thermolysin. This may not be unusual considering that tetanus and botulinum neurotoxins, zinc metalloproteases with which LF has active-site homology, are unable to cleave small peptides corresponding to the cleavage sites of their targets. It has been hypothesized that three-dimensional structure plays a more important a role in target specificity than linear amino acid sequence with these neurotoxins (16). It is possible that LF may recognize important structural elements of its target rather than primary sequence, and the relatively slow cleavage of these peptides may demonstrate LF's restriction of its active site to its pertinent cellular target(s). Alternatively, in vivo conditions found within the macrophage may somehow modify

TABLE 1. Peptide substrates used to measure protease activity of anthrax LF

Cleaved peptides[a]	k_{cat}/K_m (s^{-1} M^{-1})[b]
IARR/HP/YFL	50*
FGF/LP/IYRRPAS	29.4
ELYENKPRRP/YIL	8.9
YGGFLR/RI	5*
DRV/YIHP/FHL	0.3*
ELAGAPPEPA	NR
YFLFRPRN	NR
EGLPPRPKIPP	NR
LRRASLG	NR
SYSMEHFRWG	NR
IVPFLGPLLGLLT	NR
GWTLQSAGYLLGPNNFFGLM	NR
ESPLIAKVLTTEPPIITPVRR	NR
HDMNKVLDL	NR
HLGLAR	NR

[a] A slash indicates a known cleavage site as determined by mass spectrometry or peptide sequencing. Peptides were initially chosen at random until the peptide FGFLPIYRRPAS was found to cleave. From then on, peptides were selected for their potential to react with LF.

[b] An asterisk indicates that the k_{cat}/K_m value is estimated. NR, no reaction detected. Kinetics were determined at 25°C with 500 μM substrate and 23 ng of LF/ml (250 nM), and values are averages of two experiments.

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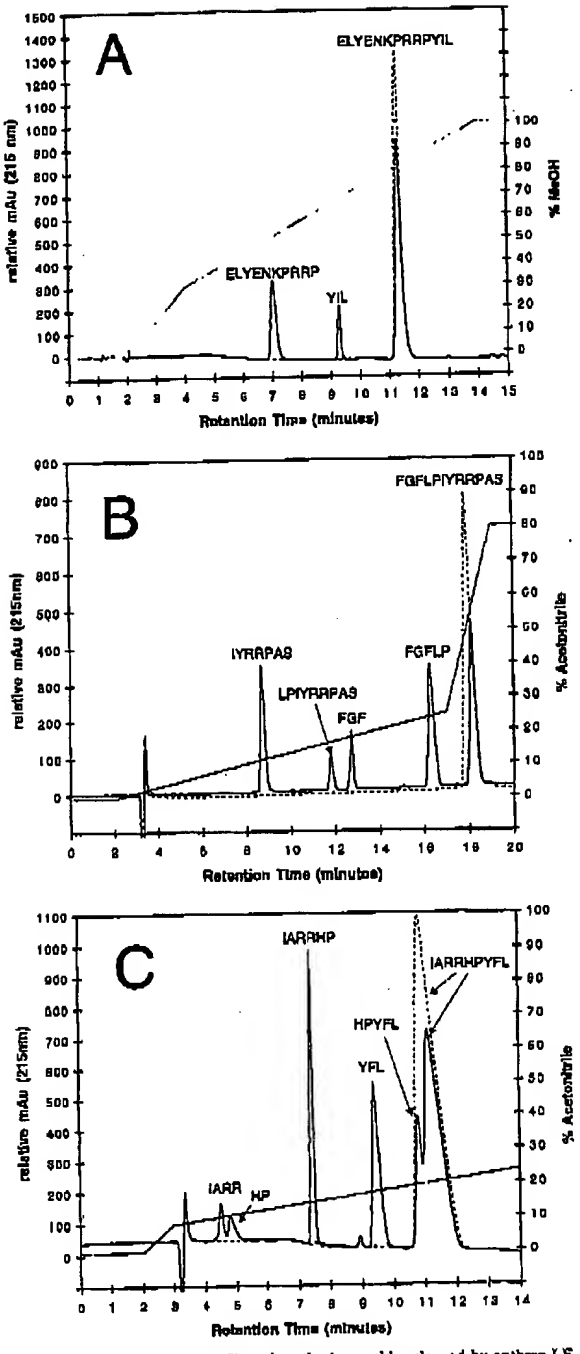

FIG. 1. HPLC elution profiles of synthetic peptides cleaved by anthrax LF. The dashed line represents 500 μM substrate alone without LF, and the solid black line represents substrate plus LF. The gray line indicates percent mobile phase. All reactions occurred at pH 7.0 in 25 mM phosphate buffer. (A) RP-HPLC elution profile of the reaction with peptide 1 (ELYENKPRRPYIL) and 23 ng of LF per ml after 18 h at 37°C. The HPLC running buffer contained 25 mM potassium phosphate (pH 7.5), 2% MeOH, and 2% tetrahydrofuran. (B) Elution profile of the reaction with peptide 2 (FGFLPIYRRPAS) and 250 ng of LF per ml after 4 h at 37°C. The HPLC running buffer was 0.1% TFA in water. The mobile phase was 0.1% TFA in acetonitrile. (C) Elution profile of the reaction with peptide 3 (IARRHPYFL) and 250 ng of LF per ml after 90 min at 37°C. The HPLC running buffer was 0.1% TFA in water. The mobile phase was 0.1% TFA in acetonitrile. All reactions were monitored by UV absorption at 215 nm (see text for more details). The HPLC flow rate was 1.0 ml/min. These results are typical examples of experiments repeated many times.

TABLE 2. Chelator and protease inhibitor profile of anthrax LF

Metal chelator or protease inhibitor	Concn	% Initial activity[a]
Metal chelators		
EDTA	10 mM	0
EDTA	5 mM	6.9
EGTA	5 mM	19.7
1,10-Phenanthroline	1 mM	0
Protease inhibitors		
Alanine hydroxamate	500 μM	>99
Arginine hydroxamate	1 mM	>99
Glutamate γ-hydroxamate	10–500 μM	>99
Glycine hydroxamate	500 μM	>99
Isoleucine hydroxamate	500 μM	>99
Leucine hydroxamate	350 μM	50
Phenylalanine hydroxamate	300 μM	50
Tyrosine hydroxamate	650 μM	50
ZINCOV	350 μM	50
Amastatin	100 μM	>99
Aprotinin	0.05 U	>99
L-Arginine	500 μM	>99
Bestatin	50 μM–1 mM	>99
Calpeptin	75 μM	>99
Captopril	1 mM	>99
Lysine CMK	0.1–1 mM	>99
Nitrobestatin	10 μM	>99
N-Succinyl-L-Proline	100 μM	>99
PMSF[b]	100 μM	>99
Phosphoramidon	500 μM	>99
Tosyl lysine CMK	100 μM	>99
Tosyl phenylalanine CMK	10 μM	>99
Trifluoroacetyl LysPro	100 μM	>99
Sodium dodecyl sulfate	1%	0

[a] A 500 μM concentration of peptide 1 (ELYENKPRRPYIL) was mixed with 250 nM of LF mutant and allowed to react for 18 h at 37°C. Samples were then analyzed on an HPLC as described in Materials and Methods. The areas of the substrate and product peaks were measured and compared to that of substrate controls. The results are averages of duplicate experiments.

[b] PMSF, phenylmethylsulfonyl fluoride.

LF to a more active form (e.g., phosphorylation and nicking, etc.).

Inhibitor profiles. LF cleavage of peptides was completely inhibited by the addition of either 1 mM 1,10-phenanthroline or 10 mM EDTA, both of which chelate zinc (Table 2). Activity was partially inhibited by 5 μM EGTA. This indicates that certain metal ions are essential for LF activity in vitro. LF that was cleared of metals through dialysis with EDTA and phenanthroline (see Materials and Methods) showed no propensity to cleave peptides. As individual metals are added back to LF, it is clear that both zinc and calcium are essential for full protease activity (Fig. 2). Additionally, specific amino acid hydroxamates, selective inhibitors of zinc metalloproteases, have been shown to inhibit LF both in vitro and in vivo. Amino acid hydroxamates are reversible inhibitors that fit the enzyme's active site while chelating the zinc ion (3). Tyrosine and leucine hydroxamate showed the best in vitro inhibition with 50% inhibitory concentrations of approximately 300 and 350 μM, respectively. Additionally, ZINCOV, a novel hydrox-

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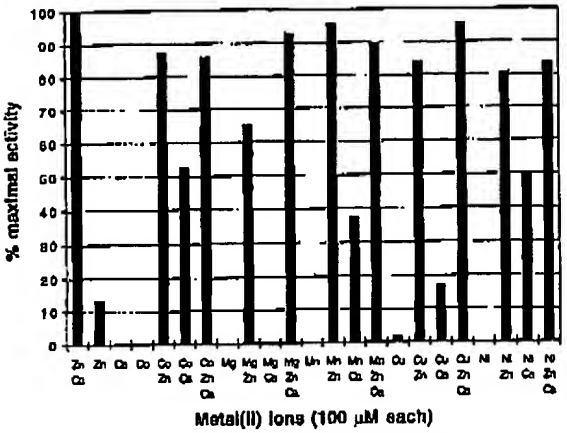

FIG. 2. In vitro protease activity of metal-reconstituted anthrax LF. Comparison of the areas of the major product peaks (see Fig. 1A, peak labeled ELY ENKPRRP) created from the reaction with peptide 1 (ELYENKPRRPYIL) and LF. Reactions of 50 ng of LF per ml with 1 mM ELYENKPRRPYIL occurred at pH 7.0 in 25 mM potassium phosphate buffer containing a 100 μM concentration of the indicated metal(s). Reactions were monitored as described in the text. All metals were chloride salts, except for zinc and nickel which were sulfate salts. After 9 h at 37°C, reaction mixtures were injected into the HPLC. It is important to note that the first three columns indicate the need for both zinc and calcium ions for full catalysis. No reaction was ever observed in the absence of metals, or with calcium alone, cobalt alone, magnesium alone, manganese alone, or nickel alone. The fastest reaction consistently was that in which only zinc and calcium were added. These results are an average of two experiments.

amate, inhibits LF in vitro protease activity at 350 μM and completely protects macrophages in vivo at a concentration of 500 μM (in vivo data not shown). The ability of hydroxamates to inhibit LF supports LF having zinc metalloprotease activity. Further, the specific ability of tyrosine and leucine hydroxamates to inhibit LF may implicate these amino acids as important for active site binding. Previously, relatively high concentrations (200 μM) of the protease inhibitors bestatin and lysine CMK were shown to protect cultured macrophages from lysis by anthrax LeTx (10). In contrast, bestatin and lysine CMK did not inhibit in vitro LF proteolysis of peptides 1 and 2 at concentrations ranging from 50 μM to 1 mM (Table 2), suggesting that the protective effect of these inhibitors observed with LF-challenged cultured cells might not be due to direct inhibition of the toxin's enzymatic activity but rather to some other event in the cytolytic cascade. As an example of this phenomenon, in lipopolysaccharide-stimulated monocytes, inhibitors of metalloproteases were observed to block maturation and release of shock-inducing cytokines (15). It is possible that bestatin and lysine CMK inhibit an event downstream from initial target cleavage by LF. Other classes of protease inhibitors, such as phenylmethylsulfonyl fluoride, *N*-succinyl-L-proline, tosyl lysine CMK, tosyl phenylalanine CMK, and nitrobestatin, did not inhibit LF activity in vitro (Table 2).

Active-site mutations. Amino acid substitutions in $LF_{686-690}$ (the HEXXH motif) were previously shown to be incapable of killing cultured macrophages, suggesting that this region is important for cytotoxicity and perhaps is involved in catalytic function (10). To determine whether this consensus thermolysin-like active-site motif may be directly involved with cleavage of peptides, and to ensure that all hydrolytic activities observed in our assays are specific to LF, amino acid substitutions in $LF_{686-690}$ were tested for the ability to cleave peptides 1 and 2. Mutant toxin molecules assayed were LF^{E687C}, LF^{E687D}, LF^{H686A}, LF^{H690A}, and $LF^{H686A+H690A}$. The recombinant mutants proteins were purified as stable, full-length molecules as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis with high-titer polyclonal antitoxin. All histidine mutants were found to have decreased zinc binding as compared to that of wt LF and were unable to kill macrophages in LeTx assays (10; also data not shown). All mutants were mixed individually with peptide 1, 2, or 3 to determine hydrolytic activity. No significant reduction in substrate concentration or generation of cleavage product was observed by RP-HPLC even after 18 h at 37°C (Table 3). This indicates that LF^{H686} and LF^{H690} are important residues for both Zn^{2+} binding and catalysis as well as cytotoxicity. The LF^{E687C} mutant was found to bind zinc at a level equal to that of wt LF yet was also unable to kill cultured cells in LeTx assays (10; also data not shown). However, as with the histidine mutants, LF^{E687C} showed no observable cleavage of peptide 1 even after incubation for 18 h at 37°C (Table 3). Additionally, work with our mutant LF^{E687D} showed no ability to cleave peptides in vitro, even at a very high concentration of enzyme. This mutant was also unable to kill macrophages in vivo (data not shown). This indicates that LF^{E687} is important for cytotoxicity and catalysis but not for Zn^{2+} binding. Importantly, the loss of hydrolytic activities associated with point mutations in the active-site region acts as a control for assignment of hydrolytic functions to LF and not to some undefined contaminating protease.

There were other supporting data for all activities being specific to LF, namely, that LF purified from the supernatant of *B. anthracis* and recombinant LF purified from *E. coli*-soluble extract maintain identical substrate specificities and kinetics (data not shown). For these reasons, it is clear that the observed proteolytic activity is exclusively a property of LF, not of contaminating proteases.

Near the center of the primary amino acid sequence of LF (residues 315 to 416) are five homologous repeats, each 19 amino acids long (Fig. 3). These repeats were first investigated by Quinn et al. (18), who discovered that dipeptide insertions into this region resulted in unstable gene products. Recently, these repeats were proposed to form an EF-hand calcium binding motif (17a; motif reviewed in reference 17) through alignments with sequences of other EF-hand-containing proteins, such as parvalbumin, and an EF-hand consensus sequence (reviewed in reference 17). The results of current metal ion reconstitution experiments are in agreement with this EF-

TABLE 3. Proteolytic activity of anthrax LF mutants

Enzyme	% Cleavage of peptide 1[a]
LF (wt)	85.9
LF (recombinant)	82.1
LF^{H686A}	3.4
LF^{H690A}	3.2
$LF^{H686A+H690A}$	3.0
LF^{E687C}	2.4
LF^{E687D}	0
BSA[b]	0
Trypsin	100
Pronase	100

[a] A 500 μM concentration of peptide 1 (ELYENKPRRPYIL) was mixed with 23 ng (250 nM) of enzyme and allowed to react for 18 h at 37°C. Samples were analyzed on an HPLC as described in Materials and Methods. The areas of the substrate and products peaks were measured and compared to that of substrate controls (no enzyme). The results are averages of duplicate experiments.
[b] BSA, bovine serum albumin.

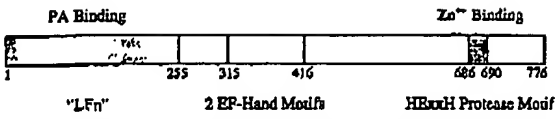

FIG. 3. Known LF domains. The first 255 amino acids are involved in binding to PA, and this region has been termed LFn. This region has a high degree of homology to the first 255 amino acids of anthrax edema factor, which also binds PA. Residues 315 to 416 contain five repeat regions that follow the consensus sequence for two EF-hand calcium-binding motifs (e.g., calmodulin). Residues 686 to 690 contain a thermolysin-like zinc metalloprotease motif HEXXH. Additionally, residues 745 to 749 (HSTDH), which are similar to the inverted zinc metalloprotease motif HXXEH (e.g., insulin-degrading enzyme), could potentially act as another zinc site, either structural or enzymatic.

hand hypothesis and clearly indicate the need for calcium as well as the catalytic zinc to achieve maximum catalysis. EF-hand motifs are the most common calcium binding motifs which are involved mainly in regulation (e.g., calmodulin) and calcium buffering (e.g., parvalbumin) (2). However, only a few EF-hand motifs have been found in prokaryotes, calerythrin being the only well-documented example (1). For this reason, gene transfer has been suggested for the existence of EF-hand motifs in prokaryotes (2). Since LF is likely to contain an EF-hand motif and since gene transfer of bacterial toxins has been a popular hypothesis, especially of ADP-ribosylation toxins (18), it is inviting to suggest that gene transfer is involved with LF. A curious coincidence is that edema factor, an adenylate cyclase that serves as another A domain in anthrax toxin, requires, as a cofactor, calmodulin, an EF-hand-containing protein (12).

B. anthracis LeTx is the major virulence factor responsible for symptoms associated with systemic anthrax (reviewed in references 5 and 11). Although LF, through its association with PA, can bind to and enter the cytoplasm of most cells tested, only macrophages seem to be affected (4). In macrophages, LF induces hyperstimulation of the oxidative burst, expression of proinflammatory cytokines tumor necrosis factor alpha and interleukin 1β, and cytolysis (6, 8). The release of these potent host mediators are responsible for the dramatic hypotension, shock, and death of the victim (6, 8, 11). It is interesting to speculate that the proteolytic activities associated with LF cleave some cytoplasmic protein responsible for regulation of macrophage inflammatory processes. The exact nature of pertinent cellular LF targets and whether proline specificity is maintained within these targets remain to be determined.

We are grateful to Sylvia Hill for providing expert technical assistance and to Carlo Petosa, Terry Dixon, and John Ireland for useful discussions.

Editor: J. T. Barbieri

This study was supported in part by National Institutes of Health grants AI08649 and AI40644, American Cancer Society grant ACS-IRG158K, and funds from the Duke University Medical Center.

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The Pathology of Experimental Anthrax in Rabbits Exposed by Inhalation and Subcutaneous Inoculation

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• *Objective.*—Although rhesus monkeys are considered to be an appropriate model for inhalational anthrax in humans, an alternative for vaccine and therapeutic efficacy studies is desirable. This study characterized the pathology of lethal anthrax in rabbits challenged by subcutaneous inoculation and aerosol exposure.

Materials and Methods.—New Zealand white rabbits were exposed by subcutaneous inoculation or aerosol to lethal doses of *Bacillus anthracis* spores.

Results.—The pathology of anthrax in rabbits exposed by either route was similar, with principal findings occurring in the spleen, lymph nodes, lungs, gastrointestinal tract, and adrenal glands. The cardinal changes were hemorrhage, edema, and necrosis, with bacilli and limited leukocytic infiltration. Features that depended on the route of exposure included mediastinitis in aerosol-exposed rabbits, a primary dermal lesion after subcutaneous inoculation, and differences in the pattern of lymph node involvement. Lesions observed in rabbits were comparable to those of inhalational anthrax in humans and rhesus monkeys. Noteworthy differences included the lack of leukocytic infiltration in brain and meningeal lesions, the relatively mild mediastinal lesions, and a lower incidence of anthrax-related pneumonia in rabbits compared with humans. These differences may be attributed to the greater susceptibility of rabbits to anthrax. Increased susceptibility is associated with both reduced leukocytic response to the bacilli and a more rapid progression to death, which further limits development of leukocytic infiltrates in response to the basic lesions of hemorrhage and necrosis. Primary pneumonic foci of inhalational anthrax, which may be influenced by preexisting pulmonary lesions in humans, were not observed in our rabbits, which were free of preexisting pulmonary disease.

Conclusion.—Anthrax in rabbits may provide a useful model for evaluating prophylaxis and therapy against inhalational anthrax in humans.

(*Arch Pathol Lab Med.* 1998;122:982–992)

Anthrax, caused by the gram-positive, spore-forming bacterium *Bacillus anthracis*, is primarily a disease of domestic herbivores, but it also occurs in humans as an infrequent zoonosis, typically acquired from contact with contaminated wool, hides, or meat. The three major forms of the human disease—cutaneous, inhalational, and gastrointestinal—reflect the route of entry of spores, which can occur through intradermal inoculation, inhalation, or ingestion, respectively.[1] Each form of the disease can progress to fatal systemic anthrax.

B. anthracis has long been recognized as a potential biological warfare or terrorist threat agent, and recent events in the Persian Gulf have further emphasized the need to develop adequate countermeasures against inhalational anthrax as a weapon of mass destruction.[2,3] Depending on both proximity to an effective delivery system and environmental factors affecting dispersal of the spores, the potential exists for high-dose inhalational exposure under such a scenario. During the past several years, considerable research has been devoted to the development of preexposure and postexposure anthrax prophylaxis by experimental human anthrax vaccines and antibiotic regimens.[1,4–11] The majority of these efforts use rhesus monkeys or guinea pigs as the animal models of choice for efficacy studies. In addition, the Food and Drug Administration (FDA) requires the guinea pig potency test before release of each lot of the human anthrax vaccine adsorbed currently licensed for use in the United States (21 CFR 620.23). As animal models, however, rhesus monkeys and guinea pigs have a number of disadvantages. Although rhesus monkeys are considered to be an appropriate model for inhalational anthrax in humans,[12,13] nonhuman primate use is beset by practical considerations, including the monetary investment in individual animals, intensive husbandry requirements, and safety issues incident to handling of the animals. Recent studies indicate that guinea pigs are not an accurate predictor of vaccine efficacy in nonhuman primates.[8,9,6–10] Guinea pigs, in contrast to rhesus monkeys, are difficult to protect by immunization with anthrax vaccine adsorbed, and they exhibit considerable variation in survival after subsequent challenge by virulent strains of *B. anthracis*. An alternative is needed for

Accepted for publication June 21, 1998.

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In conducting this research, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH publication 86-23, revised 1985).

The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

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efficacy studies against inhalational anthrax. Since inhalational anthrax in humans is virtually 100% fatal and treatment is rarely successful, an animal model that is highly sensitive to lethal infection was deemed most appropriate.

Initial studies were conducted to define the subcutaneous and aerosol median lethal doses (LD_{50}) for *B. anthracis* spores of the Ames strain in rabbits. Subsequent studies were performed to determine the efficacy of anthrax vaccine adsorbed and alternative vaccine candidates against anthrax induced in rabbits by inhalation or subcutaneous exposure. Preliminary data suggest that rabbits are similar to nonhuman primates in their ability to be protected by the current FDA-licensed human vaccine. Near-100% protection against lethal aerosol challenge was achieved with use of an abbreviated vaccination schedule in both species (M.L.M.P., unpublished data, 1996). Given preliminary evidence that the human anthrax vaccine appears to be efficacious against inhalational anthrax in rabbits, and in the interest of validating efficacy trials conducted in rabbits, we sought to determine how well the disease pattern in the animal model, as reflected by the terminal pathology, approximates the disease in humans. In this article, we describe the terminal pathology of lethal experimental anthrax developed in rabbits used in LD_{50} and preliminary vaccine efficacy studies, and we compare these findings to those for inhalational anthrax in humans.

MATERIALS AND METHODS

Animals

Necropsy specimens were obtained from lethally infected, nonvaccinated, male and female New Zealand white rabbits (*Oryctolagus cuniculus*) that had died after subcutaneous inoculation with (19 rabbits) or aerosol exposure to (22 rabbits) *B. anthracis* spores of the Ames strain (Table 1). Animals were observed for survival at least twice daily during the daylight phase of the photoperiod, for 21 to 28 days after exposure. Rabbits were identified by cage card and/or by subcutaneously implanted microchips and were housed individually in stainless steel rabbit cages in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Rabbits were fed once daily with commercial rabbit chow. Tap water was provided ad libitum.

Spore Preparation

The virulent Ames strain of *B. anthracis* was obtained from the US Department of Agriculture, Ames, Ia. It was grown in Leighton-Doi medium, and spores were harvested and washed in sterile distilled water as described elsewhere.[4] The spores were purified by centrifugation through 58% Renografin-76, washed again, resuspended in 1% phenol and stored at 4°C.

For subcutaneous inoculation, spores were suspended in sterile water for injection and then heat shocked at 60°C for 45 minutes. Appropriate dilutions were prepared, to achieve the desired dose of spores in a final volume of 0.5 mL/dose. Spore dilutions were held on ice until administered. Actual spore counts in the inoculum were verified by quantitative bacterial culture. Rabbits were inoculated with 0.5 mL of the material (dose range, 43 to 1.56×10^5 colony-forming units (CFU); subcutaneous $LD_{50} = 1.56 \times 10^5$ CFU; subcutaneous lethal dose$_{99}$ (LD_{99}) = 2.83×10^6 CFU) in the dorsal interscapular region.

For aerosol exposure, spores were suspended to appropriate starting concentrations in sterile water for injection, and were then heat shocked at 60°C for 45 minutes. Eight-milliliter aliquots of appropriate dilutions of spores were used for aerosol exposure, with a 3-jet Collison nebulizer with a head-only box and muzzle-only exposure used as described elsewhere.[14,15] The concentration of spores in the aerosol (sampled in water in an all-glass impinger) and the aerosol-inhaled dose were determined as described elsewhere.[14,15] The aerosol-inhaled dose ranged from 8.34×10^4 to 1.03×10^7 CFU *B. anthracis* (aerosol $LD_{50} = 1.05 \times 10^5$ CFU; aerosol $LD_{99} = 1.36 \times 10^5$ CFU).

Table 1. Anthrax in Rabbits: Exposure and Survival

Animal Number	Sex	Dose (CFU)*	Dose ($\times LD_{50}$)†	Route‡	Day of Death
1	M	43	0.03	S	3
2	M	43	0.03	S	4
3	M	4300	2.76	S	3
4	M	4300	2.76	S	3
5	M	4300	2.76	S	3
6	F	4300	2.76	S	4
7	M	16 050	10.29	S	3
8	F	16 050	10.29	S	3
9	F	16 050	10.29	S	3
10	F	33 250	21.31	S	2
11	F	33 250	21.31	S	2
12	F	156 000	100.00	S	2
13	F	156 000	100.00	S	2
14	F	156 000	100.00	S	3
15	F	156 000	100.00	S	3
16	M	156 000	100.00	S	3
17	F	156 000	100.00	S	3
18	F	156 000	100.00	S	3
19	M	156 000	100.00	S	3
20	M	83 400	1.52	A	3
21	F	83 400	1.52	A	3
22	M	107 000	1.95	A	2
23	M	114 000	2.07	A	2
24	M	466 000	8.47	A	2
25	F	500 000	9.09	A	3
26	F	606 000	11.02	A	3
27	F	694 000	12.62	A	2
28	F	740 000	13.45	A	3
29	F	874 000	15.89	A	2
30	F	886 000	16.11	A	2
31	F	920 000	16.73	A	3
32	M	4 540 000	43.24	A	3
33	M	6 340 000	60.38	A	2
34	M	6 400 000	60.95	A	2
35	M	6 660 000	63.43	A	2
36	M	7 060 000	67.24	A	3
37	M	7 400 000	70.48	A	2
38	M	8 060 000	76.76	A	2
39	M	8 460 000	80.57	A	2
40	M	8 660 000	82.48	A	2
41	M	10 300 000	98.10	A	2

* CFU indicates colony-forming units.
† LD_{50} indicates median lethal dose.
‡ S indicates subcutaneous; A, aerosol.

Necropsy

A complete necropsy was performed on each animal included in the pathology study. Gross findings were recorded, and the incidence of each finding was tabulated. Representative tissue specimens were selected and immersion-fixed in 10% neutral buffered formalin. Immediately before immersion fixation, lungs were inflated with 10% neutral buffered formalin.

Histopathology

Formalin-fixed tissue specimens were processed and embedded in paraffin (TissuePrep, Fisher Scientific, Fair Lawn, NJ) according to established procedures.[16] Histology sections were cut at 5 to 6 μm and stained with Harris' hematoxylin-eosin. Selected tissues were stained with Gram and Giemsa stains. Histopathologic findings were determined by routine light microscopy. Each finding, such as edema, hemorrhage, necrosis, and inflammation, was graded individually on a severity scale of 1 (minimal) to 5 (severe) on the basis of estimates of distribution and extent of

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Table 2. Anthrax in Rabbits: Incidence of Principal Gross Pathologic Findings by Route of Exposure

Organ and Finding(s)*	Subcutaneous (n = 19)	Aerosol (n = 22)
Lymph nodes		
Mandibular, HEM	0	13
Axillary, HEM	4	1
Inguinal, HEM	0	3
Splenomegaly	19	18
Sacculus rotundus, HEM	11	5
Cecal appendix, HEM	11	5
Lung, congestion		
ED	8	9
HEM	4	2
Adrenal, HEM	8	12
Ovary, HEM	4 (n = 11)	2 (n = 8)
Stomach, HEM, ED	2	5
Skin, inoculation site		
ED	19	NA
HEM	17	
Ventral cervical ED	3	4
Axillary ED	6	0
Epistaxis	3	10

* HEM indicates hemorrhage; ED, edema.

involvement within examined sections. The incidence of involvement for each organ or tissue was determined as the number of animals that had one or more of the principal histologic findings attributable to anthrax in that tissue. The severity index was calculated as the sum of severity scores for individual findings in an organ, divided by the number of animals in which that organ was examined histologically.

RESULTS

Clinical Observations

Rabbits included in the pathology study died 2 to 4 days after exposure to *B. anthracis* spores, with mean survival times of 2.9 days and 2.4 days for subcutaneously inoculated and aerosol-exposed rabbits, respectively. Although there was a trend for decreased survival time with increasing dose, the effect was minimal (Table 1). Fulminant disease appeared to be an all-or-none response, and no protracted illness was observed, regardless of the dose. Clinical signs were not generally apparent until within 24 hours of death, at which time rabbits became progressively lethargic and weak. Several rabbits, later found to have brain or meningeal lesions, exhibited brief periods of excitation and hyperactivity within hours or minutes before death.

Gross Pathologic Findings

In general, gross findings were similar, regardless of the route of infection. The most significant alterations occurred in the lymph nodes, spleens, lungs, adrenal glands, and gastrointestinal tracts of both groups of rabbits (Table 2). Hemorrhage and edema were the most prevalent changes within affected tissues. The most readily apparent differences between the two groups occurred in the pattern of lymph node involvement, in alterations at the dorsal scapular site of subcutaneous inoculation, and in changes at the axillary region of subcutaneously inoculated rabbits.

In subcutaneously inoculated rabbits, axillary lymph node involvement predominated, whereas mandibular lymph node involvement was most apparent in aerosol-exposed rabbits. Affected lymph nodes varied from mildly reddened to frankly hemorrhagic. Mediastinal nodes were obscured by adipose tissue and generally were not examined grossly. In the majority of rabbits, the spleen was dark red, was congested, was enlarged up to three times normal size, and had rounded edges. Petechiae and ecchymoses were commonly visible through the serosal surface of the sacculus rotundus and cecal appendix in both groups, with increased frequency among subcutaneously inoculated rabbits. Hemorrhage, also commonly observed in the adrenal glands and ovaries of rabbits in both groups, occurred less frequently in the Peyer's patches of the ileum, lungs, and meninges of the brain. The lungs of approximately 40% of rabbits in each group exhibited a lobular pattern of dark red mottling due to congestion and edema throughout all lung lobes. Hydrothorax and mediastinal hemorrhage were observed in only 2 and 1 of 22 aerosol-exposed rabbits, respectively, and were absent in subcutaneously inoculated rabbits. In all subcutaneously inoculated rabbits, the site of inoculation was thickened by dermal and subcutaneous edema, commonly accompanied by hemorrhage. Edema was frequently evident in the axillae of subcutaneously inoculated rabbits, as a gelatinous thickening of subcutaneous tissues. Edema of the ventral cervical to submandibular region was observed in fewer than 20% of the animals in both groups. In a small number of rabbits from each group, the mucosal surface of the stomach was mildly thickened by scattered foci of edema, up to 1 cm in diameter, with central hemorrhage. Multifocal petechiae occurred in the renal cortex of 1 aerosol-exposed rabbit. In addition to the changes noted above, all rabbits exhibited congestion of multiple organs.

Histopathologic Findings

A summary of incidence and relative severity of the principal histopathologic findings is presented in Table 3. After subcutaneous inoculation with or aerosol exposure to *B. anthracis* spores, the most consistent histopathologic findings occurred in lymphoid tissues, including the lymph nodes, spleen, and gut-associated lymphoid tissues of the sacculus rotundus and cecal appendix.

Lymph node lesions generally presented as hemorrhagic lymphadenitis (Figures 1 and 2). The principal morphologic features were lymphoid necrosis and depletion, bacilli within sinuses and depleted cortical and paracortical areas, hemorrhage, fibrinoid necrosis of vessels, edema, and variable infiltration by heterophils. In more-severely affected lymph nodes, the normal architecture was nearly obliterated by necrosis and hemorrhage. Although the morphologic characteristics of lymph node lesions were essentially the same in both groups of rabbits, the pattern of lymph node involvement and the relative severity of lesions depended on the route of exposure. Mediastinal nodes were the most frequently affected lymph nodes in both groups of rabbits; however, the mean lesion severity was greater for aerosol-exposed rabbits. Axillary node involvement occurred with greater incidence and severity among subcutaneously inoculated rabbits, whereas submandibular node involvement was more prominent among those exposed by aerosol. Mesenteric node involvement was similar between the two groups.

Acute fibrinous splenitis, seen in all rabbits, was characterized by numerous heterophils, extremely large numbers of bacilli, and fibrin deposition throughout the red

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Table 3. Anthrax in Rabbits: Principal Histopathologic Findings by Route of Exposure

Organ and Finding(s)*	Subcutaneous†		Aerosol†	
	IN	SI	IN	SI
Lymph nodes				
Mediastinal, NEC, INF, HEM	17/18	6.33	22/22	8.09
Mandibular, NEC, INF, HEM	10/15	1.73	18/22	3.18
Mesenteric, NEC, INF, HEM	9/15	1.67	13/22	2.82
Axillary, NEC, INF, HEM	14/18	5.56	9/21	1.05
Inguinal, NEC, INF, HEM	6/18	0.56	7/22	0.82
Spleen, NEC, INF, HEM	19/19	9.00	22/22	7.91
Sacculus rotundus, NEC, HEM	14/19	3.89	9/22	1.54
Cecal appendix, NEC, HEM	13/18	4.27	11/21	1.76
Peyer's patches, NEC, HEM	6/12	1.75	2/13	0.23
Thymus, NEC	5/19	0.74	3/22	0.27
Lung, ED, ALV, INF	18/19	5.47	21/22	5.36
Mediastinum, INF, HEM	0/19	0.00	8/22	0.95
Adrenal, HEM	18/19	9.00	16/22	2.27
Kidney, tubular NEC, HEM	16/19	1.63	12/22	0.95
Ovary, HEM	4/10	1.40	5/8	1.62
Stomach, HEM, ED	4/19	0.95	4/22	0.59
Brain/meninges, HEM	5/19	1.32	4/22	0.45
Bone marrow, INF	13/19	1.68	9/22	0.64
Circulatory CON, LEUK, FIB, BAC	19/19	NG	22/22	NG
Inoculation site, INF, ED, HEM	10/11	6.82	NA	

* NEC indicates necrosis; INF, inflammation; HEM, hemorrhage; ED, edema; ALV, alveolar flooding; CON, congestion; LEUK, leukocytosis; FIB, fibrin; and BAC, bacillemia.

† IN indicates incidence (number of rabbits with one or more of the findings listed for an organ, divided by the number of rabbits in which the organ was examined histologically); SI, severity index (sum of the lesion severity scores, divided by n); NG, not graded; and NA, not applicable.

pulp; necrosis and depletion of the white pulp; multifocal hemorrhage; and diffuse congestion (Figure 3).

Lesions within other lymphoid tissues were comparable between the two groups, although a slight reduction in incidence and severity of changes in the sacculus rotundus and cecal appendix occurred after aerosol exposure. Changes in lymphoid follicles of the sacculus rotundus and cecal appendix (Figure 4) were similar to those occurring in lymph nodes. Peyer's patches of the ileum were also similarly affected, although with decreased incidence and severity. Changes in the thymus were relatively mild and included small scattered foci of lymphoid necrosis and depletion, with bacilli, edema, and minimal to mild hemorrhage. Necrosis of bronchus-associated lymphoid tissue occurred in 1 rabbit in each group.

We observed significant pulmonary changes in both groups of rabbits, and—with the exceptions of interstitial pneumonia in 2 of 22 aerosol-exposed rabbits and pleuritis in 1 of 19 subcutaneously inoculated rabbits—these changes were the same between the two groups. Typically, there was distention of alveolar capillaries by congestion and large numbers of bacilli, large masses of bacilli enmeshed in fibrin within larger pulmonary vessels, interstitial edema, flooding of alveolar spaces by eosinophilic edema fluid, and minimal to mild perivascular and peribronchiolar infiltrates of heterophils (Figure 5). Pulmonary hemorrhage was confirmed histologically in 1 aerosol-exposed rabbit but was not found in any subcutaneously inoculated rabbits. Acute interstitial pneumonia occurred in 2 of the 22 aerosol-exposed rabbits but in none of the subcutaneously inoculated rabbits. In the rabbits with pneumonia, alveolar septa were mildly thickened by infiltrates of heterophils, in addition to the typical pulmonary changes noted above. Acute pleuritis, observed in only 1 subcutaneously inoculated rabbit, was characterized by mild expansion of the visceral pleura by infiltrates of heterophils, bacilli, and edema.

Acute mediastinitis was observed occasionally in aerosol-exposed rabbits and, when present, was always accompanied by severe changes within associated mediastinal lymph nodes (Figure 6). In the most severely affected animals, there was infiltration of mediastinal connective tissues by moderate numbers of heterophils, with hemorrhage, fibrin, edema, and bacilli. We occasionally noted edema, hemorrhage, and bacilli within tissues immediately surrounding affected lymph nodes of subcutaneously inoculated rabbits, but changes limited to the lymph node capsule without extension into surrounding fibroadipose tissue, as was seen in aerosol-exposed rabbits, were not considered sufficient to warrant a diagnosis of mediastinitis.

Hemorrhage occurred in multiple tissues, in addition to those described above, in both groups of rabbits. Hemorrhages were not accompanied by hemosiderosis or inflammation, although fibrinoid vascular necrosis was occasionally present. The adrenal cortex, renal cortical tubules and/or glomeruli, ovaries, and subcutaneous inoculation site were frequently affected. Within the kidneys, glomerular capillaries were often distended by masses of bacilli. Multifocal hemorrhages into uriniferous spaces appeared to have drained into associated renal tubules, which contained blood and small numbers of bacilli and were lined by degenerate to necrotic epithelial cells (Figure 7). Sporadic hemorrhagic foci were present in numerous other tissues, including the neuropil, meninges, and ventricles of the brain (Figure 8); superficial gastric mucosa; ocular structures (ciliary body, iris, and optic nerve); and myocardium.

Within the femoral bone marrow, there were small foci of depletion of hematopoietic elements with infiltration by low numbers of heterophils and aggregates of bacilli (Figure 9). In addition to the rabbits with myelitis, 1 rabbit in each group exhibited aggregates of bacilli in the marrow, without an apparent leukocytic response.

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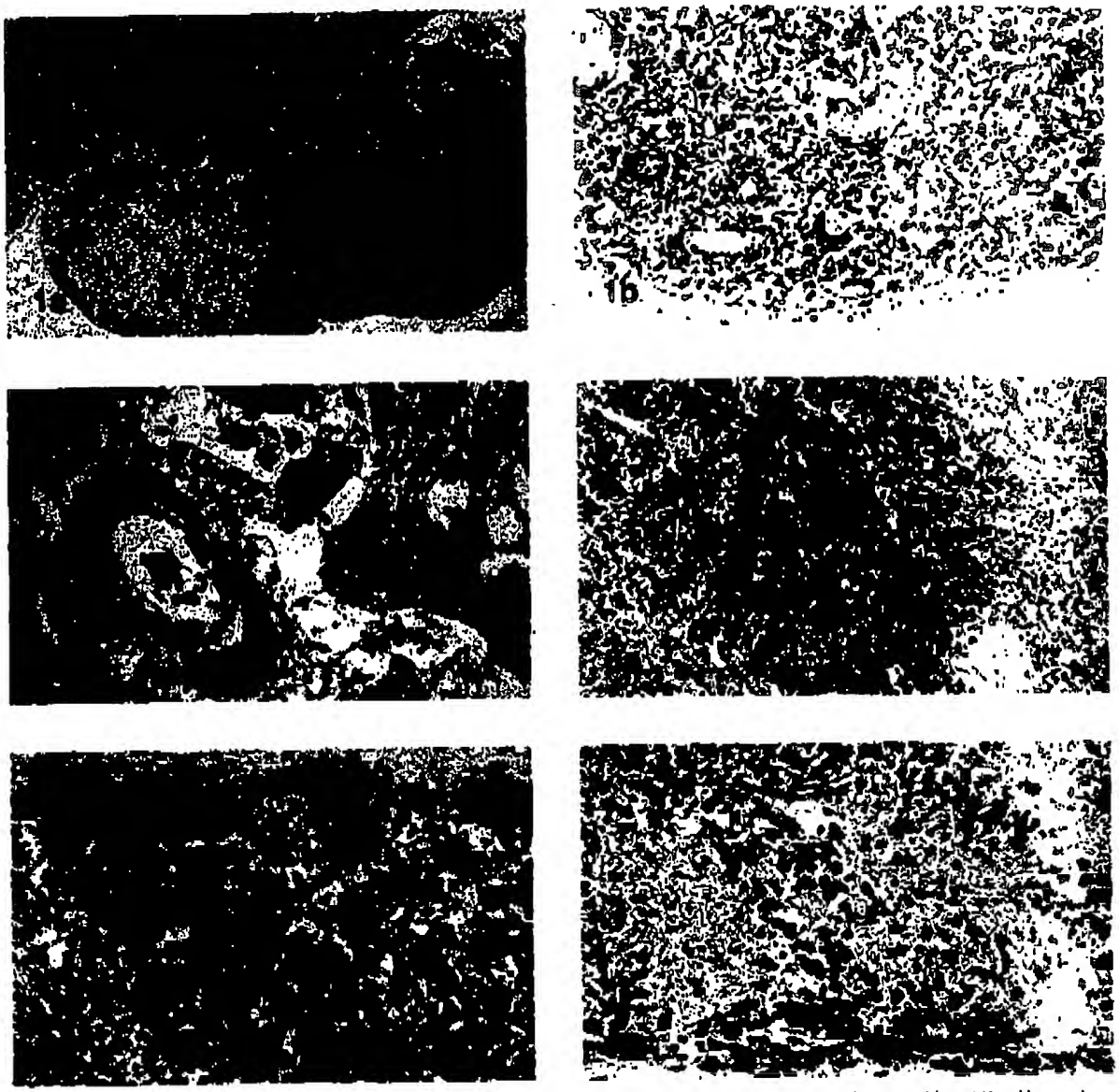

Figure 1. *Mediastinal lymph node of a subcutaneously inoculated rabbit. A, Note severe depletion of lymphoid tissue, with multifocal hemorrhage (hematoxylin-eosin, original magnification ×13.2). B, Massive numbers of bacilli are evident within the subcapsular sinus and depleted cortical areas (Giemsa, original magnification ×132).*

Figure 2. *Axillary lymph node of a subcutaneously inoculated rabbit, showing fibrinoid vascular necrosis, lymphoid necrosis, depletion, and hemorrhage within medullary cords and bacilli within medullary sinuses (hematoxylin-eosin, original magnification ×132).*

Figure 3. *Spleen of a subcutaneously inoculated rabbit. A, Note lymphoid necrosis, depletion, and hemorrhage of the lymphoid follicle. There are (B) aggregates of fibrin and (C) numerous bacilli within the red pulp (hematoxylin-eosin, original magnification ×132 [A], ×132 [B], and ×264 [C]).*

All animals in both groups developed bacillemia, intravascular aggregates of fibrin, congestion of multiple organs, and peripheral heterophilic leukocytosis. The pattern of organ involvement was the same, regardless of the route of exposure. We observed variable numbers of bacilli, often enmeshed in fibrin, within the vasculature of nearly all tissues examined. We saw increased numbers of circulating heterophils, including immature stages and occasional mitotic figures, in many tissues, which we interpreted as peripheral heterophilic leukocytosis with a left shift (Figure 10).

Acute dermatitis and/or panniculitis was a consistent feature at the inoculation sites (Figure 11). Lesions were characterized by infiltration of the dermis and panniculus

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by large numbers of heterophils, numerous bacilli, marked expansion of the dermis and panniculus by edema, multifocal hemorrhage and necrosis, occasional fibrinoid necrosis of the vasculature, and rare thrombosis. Epidermal vesiculation, ulceration, and eschar formation characteristic of cutaneous anthrax in humans were not observed.

COMMENT

Similarities in the pathology of inhalational anthrax in rabbits, humans, and rhesus monkeys were readily apparent. Subcutaneous exposure of rabbits also resulted in rapidly fatal septicemic disease, rather than the characteristic skin lesions and low mortality typical of cutaneous anthrax in humans. In rabbits exposed by either route, the most pathologically significant and consistent findings occurred in the spleen, lymph nodes, lungs, gastrointestinal tract, and adrenal glands. Additional lesions occurred in the mediastinum (aerosol exposure only), brain, bone marrow, kidney, thymus, heart, and ovaries. Lesions were necrotizing and hemorrhagic, generally accompanied by little local leukocytic response. The lesions of inhalational anthrax in humans are also characterized as hemorrhagic to necrohemorrhagic, with little cellular inflammatory response.[17,18] The spleen, lymph nodes, mediastinum, lungs, gastrointestinal tract, and brain are principal sites of involvement.[13,18–26] Rhesus monkeys inoculated intradermally or exposed to aerosolized spores of *B. anthracis* also develop necrotizing and hemorrhagic lesions similar to those seen in our rabbits.[12,27–29]

With few exceptions, the pathology of lethal infection by the Ames strain of *B. anthracis* in rabbits was the same whether animals were exposed subcutaneously or by aerosol. Except for the presence of a primary dermal lesion after subcutaneous inoculation and the lack of a corresponding primary pneumonic focus after aerosol exposure, the pathogenesis of anthrax induced by either route appears similar. Ross[30] demonstrated that after inhalational exposure, spores are taken up by alveolar macrophages for transport, by way of lymphatics, to intrathoracic lymph nodes. Infection begins with proliferation of the organisms in the lymph nodes rather than as a primary focus in the lungs, thus explaining the rarity of pneumonia after aerosol exposure. Studies by Lincoln et al[31] further demonstrated the role of lymphatics and intrathoracic lymph nodes in the establishment of systemic inhalational anthrax. The appearance of organisms in the lymphatics draining the lungs and the establishment of infection in the intrathoracic lymph nodes always precedes the development of bacillemia after aerosol exposure. As the phagocytic capacity of the lymph node is overwhelmed, vegetative organisms pass through efferent lymphatics, infect successive nodes, and ultimately enter the blood stream through the thoracic duct. Lincoln et al[31] demonstrated a similar sequence of events in the establishment of systemic anthrax induced by intradermal inoculation. Regional lymph nodes draining the site of inoculation are infected initially. Shortly before the onset of bacillemia, organisms can be cultured from thoracic lymph, and, ultimately, they enter the blood stream through the thoracic duct. Once bacillemia is established, the pathogenesis is the same, regardless of the route of initial exposure.

Major differences between anthrax induced by the two routes of exposure, in rabbits, were the pattern of lymph node involvement, the development of mediastinitis exclusively in aerosol-exposed rabbits, and the dermal lesion seen after subcutaneous inoculation. Similarly, Gleiser et al[29] reported that the basic nature of the lesions of inhalational anthrax was no different from that seen after intradermal inoculation in rhesus monkeys, although lesion distribution exhibited several distinguishing features. Aerosol-exposed monkeys exhibited a high incidence of mediastinitis accompanied by a high incidence of hemorrhagic meningitis, hemorrhagic pulmonary lesions, and intrathoracic lymphadenopathy. Fritz et al[12] reported a high incidence of mesenteric as well as tracheobronchial lymph node involvement in inhalational anthrax in rhesus monkeys. Intrathoracic lymphadenitis and mediastinitis are considered hallmarks of inhalational anthrax in humans and are believed to represent the primary focus of infection.[13,17,19,20,32] A low incidence of mesenteric node involvement was also reported in humans with inhalational anthrax.[19,33] In contrast to the findings for inhalational anthrax, Berdjis et al[28] described a low incidence of mediastinitis and hemorrhagic meningitis, with cellulitis at the inoculation site, and primarily axillary lymph node involvement in monkeys inoculated intradermally in the forearm.

Differences in the pattern of regional lymph node involvement reflect normal lymphatic drainage from the subcutaneous or pulmonary site of exposure. In contrast to findings in humans and rhesus monkeys, however, we observed significant mediastinal lymph node involvement in rabbits, regardless of the route of exposure. A possible explanation lies in the fact that the mediastinal lymph nodes in various species can receive afferent lymphatics from muscles of the dorsal thoracic wall and scapula, which was the site of inoculation in our rabbits, in addition to efferent lymphatics originating from the intercostal, tracheobronchial, and bronchopulmonary nodes.[34–38] Greater lesion severity with extension into mediastinal tissues of aerosol-exposed rabbits can be attributed to earlier, more direct involvement of intrathoracic nodes during the course of aerosol infection. The greater incidence and severity of axillary node involvement among subcutaneously inoculated rabbits may be similarly regarded as a consequence of lymphatic drainage from the site of inoculation, resulting in early involvement of the axillary nodes. The increased incidence and severity of submandibular node involvement occurring in aerosol-exposed rabbits may be the consequence of either direct oropharyngeal deposition or mucociliary clearance of a portion of the aerosol from distal respiratory tissues.

The mediastinal lesions we observed in aerosol-exposed rabbits were similar, although less severe than those described in humans. The decreased incidence and severity of the lesions in rabbits were most likely due to the rapid progression of systemic changes, resulting in relatively short survival times compared to survival times for humans or monkeys. In aerosol-exposed rabbits, the mean survival time was 2.4 days after exposure, with only a minimal effect of decreasing dose on prolonging survival. Specific dates of exposure are rarely known for human cases, but estimates for 41 cases from the Sverdlovsk outbreak place mean survival time at 18.5 days after exposure.[19,39] The clinical course of infection was also modified through extensive medical intervention in those cases. A more protracted course of clinical disease would allow greater opportunity for extension of the primary focus of infection from the intrathoracic nodes into adjacent mediastinal tissues.

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The effect of prolonging the time course of infection on the severity of mediastinal lesions was demonstrated experimentally in rhesus monkeys. Gleiser et al[29] found that mediastinal edema and some hemorrhage were frequent findings in aerosol-exposed monkeys, but massive hemorrhagic mediastinitis was limited to (1) an animal that died 11 days after exposure to a low aerosol dose and (2) those animals in which the course of the disease was prolonged through the use of antibiotics. In some monkeys that died within 2 to 5 days after exposure to high doses of aerosolized spores, mediastinitis was not seen and intrathoracic lymph node involvement was the only gross finding.

It is noteworthy that 2 of 10 animals in the intradermal study by Berdjis et al[28] developed mediastinitis. Although the distribution of lymph node involvement was not presented in detail, beyond citing axillary nodes as a specific site, the development of mediastinitis in these monkeys suggests that mediastinal node involvement may occur after intradermal and aerosol exposure in rhesus monkeys, as occurred in our rabbits. In the study of inhalational anthrax in rhesus monkeys, by Gleiser et al,[29] mediastinitis was interpreted to be an extension of lesions originating in the mediastinal lymph nodes. Mediastinal lesions were most intense around intrathoracic nodes, which were frequently necrotic and hemorrhagic; and, as indicated above, some monkeys dying rapidly after exposure to high doses of aerosolized spores developed intrathoracic lymph node involvement without mediastinitis. In a more recent study of inhalational anthrax in rhesus monkeys with a mean survival time of 5.5 days after exposure, Fritz et al[13] observed a gross incidence of thoracic node involvement in 46% and mediastinitis in only 30% of monkeys at necropsy. Such findings suggest that intrathoracic lymph node involvement precedes the development of mediastinitis and that mediastinitis is the result of direct extension of lesions from the lymph nodes into adjacent tissues. A similar pathogenesis for mediastinal lesions may be involved in the Berdjis study[28] of anthrax in monkeys exposed by intradermal inoculation. The mediastinitis of inhalational anthrax in humans is also described as having a perinodal distribution and was interpreted to have arisen as an extension of primary lymph node lesions.[19] Mediastinal lymph node involvement would be expected to occur early in the course of the disease after aerosol exposure. This could account for the greater severity of mediastinal node lesions we observed in our aerosol-exposed rabbits, in addition to providing increased opportunity for extension into surrounding mediastinal tissues.

The lesions we observed in the gastrointestinal tract were comparable among rabbits exposed by aerosol and subcutaneous inoculation. Gut-associated lymphoid tissues of the sacculus rotundus, cecal appendix, and ileum were primarily affected. We also noted sporadic foci of hemorrhage with bacilli and edema in the stomach, small intestine, colon, and esophagus. In humans, inhalational anthrax is also associated with gastrointestinal hemorrhage, necrosis, and edema involving the stomach, small intestine, and colon.[18–20,33,35] However, Abramova et al[19] noted that, among the Sverdlovsk cases, the intestinal lesions did not involve Peyer's patches. In contrast, other investigators reported that intestinal lesions in humans with inhalational anthrax were sometimes the result of bacilli multiplying in the gut-associated lymphoid tissues.[35]

One might attribute the presence of lesions in the gastrointestinal mucosa, gut-associated lymphoid tissues, and mesenteric lymph nodes, after aerosol exposure, to primary gastrointestinal anthrax. It is known that 65% to 70% of inhaled *B. anthracis* spores are ultimately coughed up, are swallowed, and pass into the stomach within a few hours of exposure.[40] However, the presence of identical gastrointestinal findings in subcutaneously inoculated rabbits, where ingestion of spores is unlikely, suggests that these lesions occur secondary to lymphatic or hematogenous dissemination of the bacilli. Gastrointestinal lesions were typically accompanied by distension of associated blood vessels by numerous bacilli, also consistent with hematogenous origin. Investigators who examined cases from the Sverdlovsk outbreak believed that the gastrointestinal lesions were mainly, or possibly wholly, of hematogenous origin, and they emphasized that, in 90% of all human anthrax cases involving gastrointestinal lesions, the enteric pathology occurs secondary to hematogenous dissemination from a cutaneous or respiratory site of exposure.[19] Similarly, hematogenous dissemination was considered to be the most likely origin for gastrointestinal lesions in aerosol-exposed rhesus monkeys.[13] A study by Lincoln et al[31] showed that rhesus monkeys are extremely resistant to gastrointestinal exposure to *B. anthracis*, providing additional evidence that the gastrointestinal lesions were not likely due to primary infection by ingestion.

Pulmonary lesions were observed in nearly all rabbits, regardless of the route of exposure. An interesting finding among the aerosol-exposed rabbits was the occurrence of 2 cases of acute interstitial pneumonia. Whether the pneumonia represented primary pneumonic anthrax or developed secondary to bacillemia could not be determined definitively. However, the interstitial pattern was most consistent with secondary hematogenous origin, as opposed to bacterial pneumonia of inhalational origin, which typically presents as a bronchopneumonia.[41] Fritz et al[13] reported anthrax-related pneumonia in 2 of 13 rhesus monkeys and interstitial pneumonia in a third monkey after exposure to aerosolized spores. The two cases of anthrax-related pneumonia were thought to be secondary to bacilli occluding and disrupting alveolar septal capillaries. The single case of interstitial pneumonia was thought to represent an early event in the development of anthrax-related pneumonia. The presence of pneumonitis in 3 of 10 intradermally inoculated monkeys studied by Berdjis et al[28] also supports a hematogenous route as a viable pathogenesis for the development of pneumonic anthrax. A similar pathogenesis appears likely for the development of pneumonia in our rabbits, and it may account for many of the pneumonias cited in cases of anthrax in humans.

There is evidence suggesting that the greater incidence of pneumonia among humans with inhalational anthrax might be influenced by the presence of preexisting pulmonary lesions. Case reports made before the Sverdlovsk outbreak of 1979 suggest that primary pneumonic anthrax did not occur in the absence of preexisting pulmonary disease.[13,20,42] In the Sverdlovsk outbreak, however, large-focal hemorrhagic and necrotizing pneumonia interpreted as primary bronchopneumonia was reported for 11 of 42 cases.[17,19,39] The incidence of preexisting pulmonary lesions was not specifically addressed in reports of the Sverdlovsk cases. However, epidemiologic data reported by Meselson et al[37] suggest that a significant segment of the affected population engaged in activities associated with impaired pulmonary function that may have rendered them at in-

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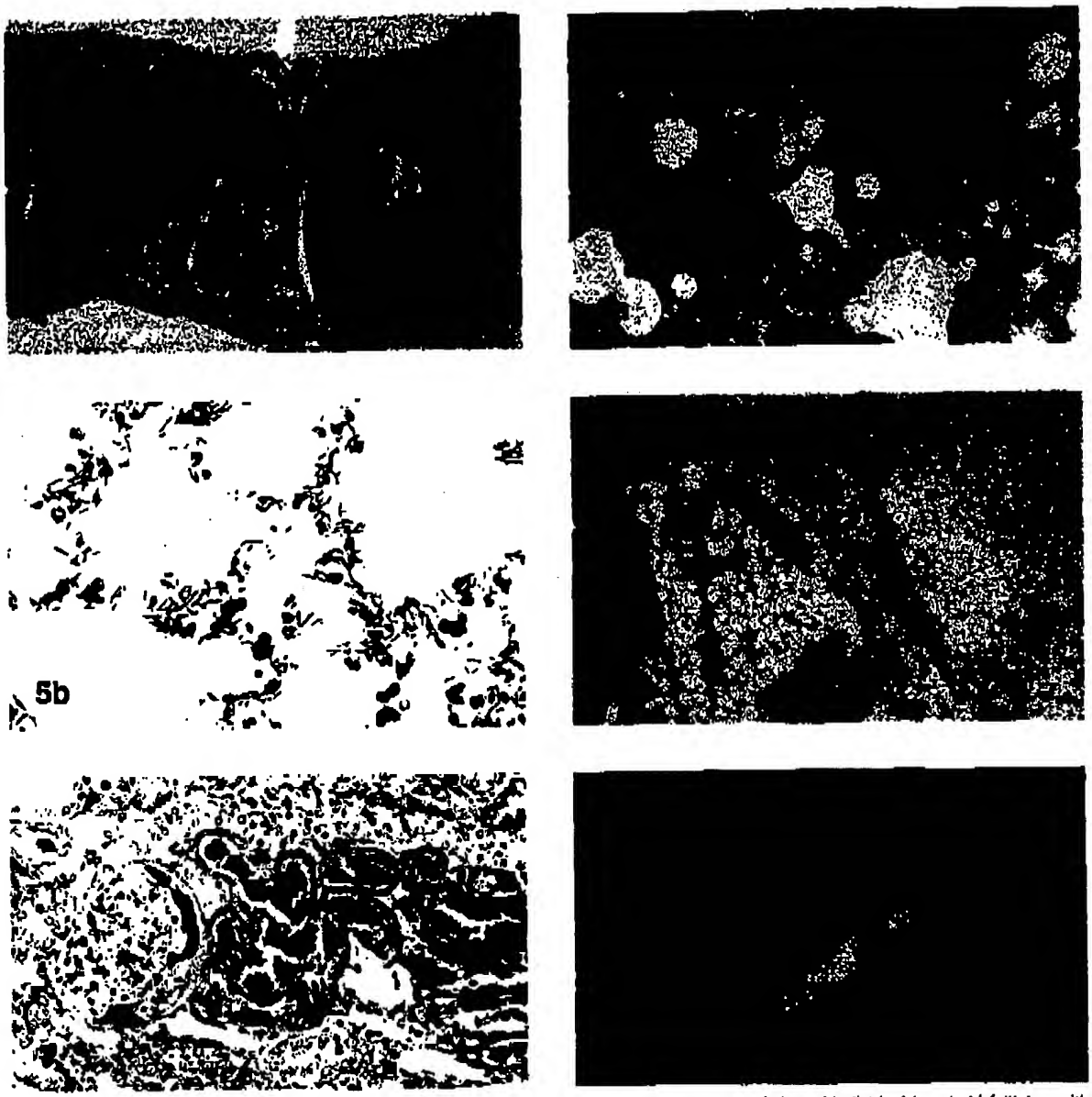

Figure 4. *Cecal appendix of a subcutaneously inoculated rabbit. There is severe necrosis and depletion of individual lymphoid follicles, with hemorrhage. Numerous bacilli infiltrate depleted foci, similar to findings illustrated in lymph nodes (Figure 1, B) (hematoxylin-eosin, original magnification ×13.2).*

Figure 5. *Lung of a subcutaneously inoculated rabbit. A, Note flooding of alveolar spaces with edema fluid and B, severe bacillemia evident in alveolar septal capillaries. Cellular inflammatory infiltrates (ie, pneumonic foci) are not apparent (hematoxylin-eosin, original magnification ×132 [A], and Giemsa, original magnification ×264 [B]).*

Figure 6. *Mediastinum of an aerosol-exposed rabbit. Note multifocal hemorrhage, edema, and necrosis extending into mediastinal fibroadipose tissue adjacent to a necrohemorrhagic mediastinal lymph node (hematoxylin-eosin, original magnification ×26.4).*

Figure 7. *Kidney of a subcutaneously inoculated rabbit. Note hemorrhage within the uriniferous space and lumina of associated convoluted tubules. Affected tubules are lined by degenerate to necrotic epithelium. Glomerular capillaries are distended by aggregates of bacilli (hematoxylin-eosin, original magnification ×132).*

Figure 8. *Cerebral cortex of a subcutaneously inoculated rabbit. Note hemorrhagic meninges and neuropil and the absence of any apparent cellular inflammatory infiltrates (hematoxylin-eosin, original magnification ×26.4).*

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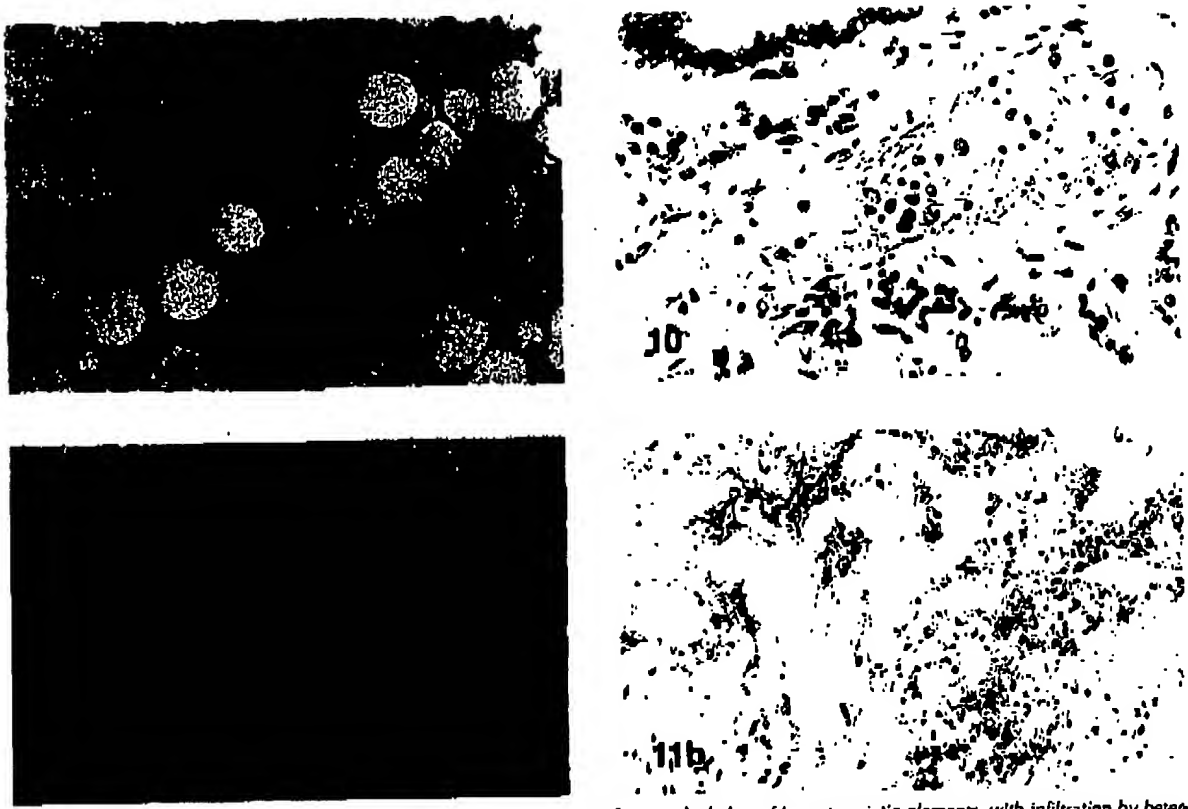

Figure 9. *Femoral bone marrow of a subcutaneously inoculated rabbit. There is depletion of hematopoietic elements, with infiltration by heterophilic granulocytes and myriad bacilli (hematoxylin-eosin, original magnification ×132).*

Figure 10. *Pulmonary arteriole of a subcutaneously inoculated rabbit. Note the extreme bacillemia and numerous heterophilic granulocytes in various stages of maturation (Giemsa, original magnification ×264).*

Figure 11. *Inoculation site of a subcutaneously inoculated rabbit. The dermis is markedly expanded by edema, hemorrhage, and necrosis. A, Note the absence of epidermal vesiculation or ulceration (hematoxylin-eosin, original magnification ×26.4). B, Dermal collagen bundles are separated by infiltrates of heterophilic granulocytes admixed with edema, fibrin, and numerous bacilli (Giemsa, original magnification ×132).*

creased risk for the development of primary pneumonic foci of anthrax. Information available on 33 of 55 tabulated cases in males indicated that 60% were moderate to heavy smokers. In addition, the most commonly reported occupation of those affected at the time of the outbreak was "welder" (7 of 35 reported). Early investigations of inhalational anthrax in rhesus monkeys described the development of necrotizing bronchiolitis believed to represent superinfection of preexisting lung mite nodules by *B. anthracis*.[27,28] Similar lesions were not observed in later studies of monkeys free of lung mites.[12] Our studies, performed in rabbits free of significant preexisting pulmonary lesions, and earlier studies in rabbits by Buchner and Barnes[29,30] also failed to reveal necrohemorrhagic pneumonic foci resembling those described in the Sverdlovsk outbreak.

An additional factor that may have influenced the low incidence of pneumonia and mediastinitis in rabbits compared with that in humans is relative host susceptibility. Anthrax studies in a variety of host species suggest that the degree of leukocytic response may be related to relative host susceptibility, with highly susceptible hosts developing a mild response, in contrast to the more intense inflammatory reaction seen in hosts resistant to anthrax.[34,44] Aerosol exposure of dogs and swine, both considered highly resistant to anthrax, resulted in hemorrhagic pulmonary lesions with intense cellular inflammatory infiltrates.[45] Bacilli were apparently contained within pulmonary tissues, as intrathoracic lymph nodes were reactive, but not infected, and systemic disease did not develop. On the basis of pneumonic changes after aerosol exposure, humans appear to be intermediate in susceptibility to anthrax, between the more susceptible rabbit and the more resistant dog and swine. Differences in local leukocytic response have also been cited between the dermal lesion of anthrax in rabbits and cutaneous anthrax in humans. In rabbits, the lesion is more edematous, and the cellular infiltrations are less intense than those seen in humans.[46]

All rabbits developed a histologically evident bacillemia, intravascular aggregates of fibrin, congestion of multiple organs, and peripheral heterophilic leukocytosis. Leukocytosis with a left shift has been reported for human cases.[13] The level of terminal bacillemia is also considered to be high in humans.[47] Morphologic changes consistent with shock, frequently noted in human cases, in-

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clude congestion of multiple organs, stasis of erythrocytes in venules and capillaries, and pulmonary edema.[19] The congestion suggests terminal hypotensive shock, consistent with cytokine-induced pathophysiologic events currently believed to contribute to the pathogenesis of anthrax. At high doses, anthrax lethal toxin is lytic to macrophages[48]; however, at sublytic doses, the toxin induces macrophages to express interleukin 1 and tumor necrosis factor.[49] At high levels, interleukin 1 and tumor necrosis factor can mediate a cascade of physiologic events culminating in fatal shock,[50-53] similar to that seen at the terminal stage of systemic anthrax. Intravascular aggregates of fibrin may be due to rapid postmortem clot formation peculiar to anthrax infection and the extreme bacillemia, or they may occur secondary to toxin- or cytokine-induced endothelial alterations favoring a procoagulant state.[53-56] Bacterial thrombi and leukocytosis are also observed in anthrax in rhesus monkeys exposed intradermally or by aerosol.[26,27,57]

Hemorrhages involving multiple tissues were common among both groups of rabbits and have been described for anthrax in humans[20,22,23,38,58] and rhesus monkeys[26,29] as well. In the majority of affected tissues in rabbits, the hemorrhage was accompanied by large numbers of bacilli. In the adrenal glands, however, few bacilli were apparent histologically. Hemorrhage in the adrenal glands varied from minimal to complete obliteration of the cortex and may have been a manifestation of Waterhouse-Friderichsen syndrome. Renal hemorrhages in our rabbits were accompanied by changes that have not been described for anthrax in humans or in nonhuman primates. The appearance of the lesion suggests that hemorrhage occurred through disruption of glomerular capillaries, with subsequent drainage from the uriniferous space into associated convoluted tubules. This resulted in tubular degeneration and necrosis, possibly hemoglobinuric nephrosis. In our study, the renal lesion was minimal to mild in severity, with only low numbers of widely scattered nephrons involved. Significant renal lesions were not a feature of anthrax in intradermally-exposed rhesus monkeys, whereas renal tubular degeneration and tubular casts were reported for inhalational anthrax in that species.[29] Significant renal pathology does not appear to be a feature of inhalational anthrax in humans, although there is a limited account of tubular degeneration and necrosis.[58]

Hemorrhagic meningitis with intense neutrophilic inflammatory infiltrates is frequently associated with inhalational anthrax in humans and nonhuman primates.[12,13,19,20,29,59] It was also reported to occur in 1 of 10 rhesus monkeys exposed intradermally.[26] However, of the 21 cases of meningeal anthrax observed in the Sverdlovsk outbreak, 8 were described as a serous leptomeningitis characterized primarily by edema of the pia mater, with only insignificant infiltration by erythrocytes, mononuclear cells, and neutrophils.[20] A low incidence of hemorrhage with bacilli occurred in the brain and meninges of subcutaneously inoculated and aerosol-exposed rabbits. The lesion in rabbits differed from that seen most often in humans or in nonhuman primates in that it was devoid of any accompanying leukocytic infiltrate. The degree of leukocytic response may have been influenced by relative host susceptibility, as we have suggested for the mediastinal, pulmonary, and dermal lesions. In addition, our rabbits were infected with the Ames strain of *B. anthracis*, whereas most earlier studies were of anthrax in nonhuman primates infected with the Vollum-189 or Vollum 1B strains. Finally, and perhaps most importantly, the rapid progression of fatal systemic changes in rabbits may have limited the opportunity for development of the leukocytic response. In this regard, rhesus monkeys with meningeal anthrax that die soon after exposure have a significantly lower cellular inflammatory response in the central nervous system than do those with longer survival times (G.M.Z., unpublished data, 1997).

Our results indicate that rabbits are extremely sensitive to lethal infection by *B. anthracis*, as evidenced by the fulminant nature of the disease and disseminated pathologic findings. The rapidly fatal course of anthrax induced by high-dose aerosol or subcutaneous exposure in rabbits could be considered disadvantageous in that products efficacious against the more protracted human illness might go unrecognized in such an animal model. However, inhalational anthrax in humans is essentially 100% fatal if left untreated, and inhaled doses in biological warfare or terrorist scenarios might well exceed those of historical occupational exposures. It would be prudent to err on the side of safety by demonstrating efficacy in such a sensitive model.

CONCLUSION

The principal lesions of anthrax were similar in rabbits after subcutaneous injection or aerosol exposure. Major differences were the pattern of lymph node involvement, the presence of mediastinitis exclusively in aerosol-exposed rabbits, and dermal lesions in subcutaneously inoculated rabbits. Although the disease is characterized by a more rapid progression in rabbits, the end-stage pathology of anthrax in the rabbit model appears remarkably similar to that of inhalational anthrax in humans, and it supports the use of rabbits as an appropriate animal model. Furthermore, the more fulminant nature of the disease in rabbits could be considered advantageous in that it provides a rigorous test of candidate products, useful in the development of vaccines and therapeutic regimens against inhalational anthrax in humans.

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PAGE 49/49 * RCVD AT 12/18/2003 6:12:43 PM [Eastern Standard Time] * SVR:USPTO-EFXRF-2/0 * DNIS:7465209 * CSID:2162410816 * DURATION (mm-ss):19-34

Molecular Microbiology (1994) 13(6), 1093–1100

(B)

Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity

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Summary

Comparison of the anthrax toxin lethal factor (LF) amino acid sequence with sequences in the Swiss protein database revealed short regions of similarity with the consensus zinc-binding site, HEXXH, that is characteristic of metalloproteases. Several protease inhibitors, including bestatin and captopril, prevented intoxication of macrophages by lethal toxin. LF was fully inactivated by site-directed mutagenesis that substituted Ala for either of the residues (H-686 and H-690) implicated in zinc binding. Similarly, LF was inactivated by substitution of Cys for E-687, which is thought to be an essential part of the catalytic site. In contrast, replacement of E-720 and E-721 with Ala had no effect on LF activity. LF bound ^{65}Zn both in solution and on protein blots. The ^{65}Zn binding was reduced for several of the LF mutants. These data suggest that anthrax toxin LF is a zinc metallopeptidase, the catalytic function of which is responsible for the lethal activity observed in cultured cells and in animals.

Introduction

The virulence of *Bacillus anthracis* depends on the production of two secreted materials: an antiphagocytic γ-linked poly-D-glutamic acid capsule and a three-component protein exotoxin (Keppie *et al.*, 1963; Smith *et al.*, 1955). The need for both products is evident from the great decrease in infectivity that occurs upon loss of either of the two large plasmids that encode these virulence factors (Uchida *et al.*, 1986; Ivins and Welkos, 1986). Furthermore, inactivation of any one of the three genes encoding the toxin proteins causes a large decrease in virulence (Cataldi *et al.*, 1990; Pezard *et al.*, 1991). The three toxin proteins interact in binary combinations to produce two toxic effects on animal cells and tissues. Lethal factor (LF) and protective antigen (PA), together designated as lethal toxin (LT), rapidly produce pulmonary oedema and death in rats and other animals (Smith and Stanley, 1962). Edema factor (EF) and PA, a combination designated oedema toxin, causes oedema when injected intradermally (Harris-Smith *et al.*, 1958). EF is an adenylate cyclase which has the unusual property of requiring the eukaryotic protein calmodulin as an essential cofactor (Leppla, 1982; 1984).

Recent studies have helped to explain how the toxin proteins interact to cause delivery of EF and LF to the cytosol (Leppla *et al.*, 1993; Arora *et al.*, 1992; Arora and Leppla, 1993). PA binds to a receptor present on many types of cells, and is cleaved after Arg-167 by furin or a protease with similar properties (Klimpel *et al.*, 1992). The release of the amino-terminal fragment of PA exposes a site to which either EF or LF binds with high affinity (Leppla *et al.*, 1986; Leppla, 1991b). The complex then enters cells and the EF and LF are translocated to the cytosol. Studies with fusion proteins (Arora *et al.*, 1992; Arora and Leppla, 1993) show that this translocation process is highly efficient and that it may be able to accommodate many different polypeptides.

The anthrax lethal toxin plays a larger role than the oedema toxin in the pathogenesis of *B. anthracis* infections. Thus, inactivation of the EF gene in *B. anthracis* reduces virulence only 10-fold, whereas inactivation of the LF gene reduces virulence more than 1000-fold (Pezard *et al.*, 1991). Lethal toxin causes pulmonary oedema in Fischer 344 rats that leads to death in as little as 38 min (Haines *et al.*, 1965; Ezzell *et al.*, 1984). Equally dramatic is the lysis within 90–120 min of certain mouse macrophage cells exposed to low concentrations of the toxin. The response of mouse macrophages is unique: no other type of cultured cell shows an acute response to the toxin (Friedlander, 1986; Friedlander *et al.*, 1993). Cell types that are not sensitive to lethal toxin have PA receptors and can respond to oedema toxin, so their resistance suggests the absence of an intracellular substrate for LF. The potency of lethal toxin on macrophages and in rats, together with the obvious analogy of LF to the 'A' subunits of other toxins (including EF) that act in the cytosol, leads to the expectation that LF also has a catalytic

Received 22 November, 1993; revised 24 May, 1994; accepted 7 June, 1994. *For correspondence. Tel. (301) 402 0370; Fax (301) 402 0396.

activity (Gill, 1978). Early attempts to identify enzymatic activities in LF were unsuccessful (Stanley and Smith, 1963). In this report we present evidence that LF is a metalloprotease.

Results and Discussion

Database searches

The computer program BLAST was used to search for sequences similar to LF residues 383–776 (Robertson and Leppla, 1986; Bragg and Robertson, 1989; Altschul *et al.*, 1990). Short regions from many proteins showed a limited similarity to LF. Three different proteins (*Bacillus stearothermophilus* neutral proteinase (thermolysin), rat soluble metallo-endopeptidase, and *Bacillus cereus* protease) each had sequences similar to LF residues 686–692 (Kubo and Imanaka, 1988; Watt and Yip, 1989; Wetmore *et al.*, 1992). We recognized this region of LF, HEFGHAV, as characteristic of zinc metallopeptidases (Jongeneel *et al.*, 1989). Using the GCG programs PILEUP and GAP, the LF amino acid sequence was aligned to several zinc metallopeptidases (Fig. 1) (Genetics Computer Group, 1991). The highest similarity was found when LF residues 686–718 were compared with residues 142–172 of thermolysin, for which the program GAP calculated an identity of 65.4% and a similarity of 88.5% when the gap weight was set to 0.0.

Zinc metallopeptidases typically contain three residues which co-ordinate a zinc atom and a glutamic acid residue that is critical for catalysis. Two of the histidine residues and the glutamic acid residue are contained in the minimal metallopeptidase zinc-binding region signature sequence HEXXH. In these metalloenzymes, the essential zinc ion is chelated by the two histidines (H) together with a third more C-terminal residue, either His, Cys, or Glu (Vallee and Auld, 1990a,b; Hase and Finkelstein, 1993). The Glu residue (E) between the two His residues acts as a nucleophile during catalysis.

A search of LF against the PROSITE database with the program MOTIFS detected a match to the zinc metalloprotease signature ([STAIV]-x(2)-H-E-[LIVMFY]-[DEHRKP]-H-x-[LIVMFYWQ]) when a single mismatch was permitted (Genetics Computer Group, 1991). The G in the first position was the only mismatch from the 10-residue motif and is chemically similar to the accepted A. The similarity of the tetanus toxin sequences to the zinc metallopeptidase family was reported when the motif was initially described (Jongeneel *et al.*, 1989; Fairweather and Lyness, 1986). When compared to this motif, tetanus toxin also has a mismatch at position 1, whereas the botulinum neurotoxin types C1 and D match exactly. Botulinum neurotoxin type A also fails to match the extended PROSITE motif owing to a single mismatch at position 10. Recently, convincing evidence was obtained that tetanus toxin and several of the botulinum toxins are proteases that bind zinc and they have been included as members of this motif family (Schiavo *et al.*, 1992b, c; 1993; Bairoch, 1993). The widely accepted view that all the clostridial neurotoxins have a similar catalytic activity suggests that all these proteins will be shown to act as peptidases.

Aminopeptidase inhibitors block lethal toxin action

We tested several inhibitors of endopeptidases and aminopeptidases for their ability to prevent intoxication of J774A.1 macrophage cells by LT. The most effective inhibitors of LT action were the chloromethyl ketones of Leu and Phe (Leu-CMK or Phe-CMK) (Fig. 2 and data not shown). Unfortunately these substances were themselves toxic at concentrations above 0.1 mM, presumably because they interfere with a number of cellular processes. Bestatin ((2S, 3R)-(3-amino-2-hydroxy-4-phenylbutanoyl)-L-leucine),

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                  *  *                                        **
Lethal factor   686 HEFGHAVDD Y.AGYLLDKN Q...SDLVTNS KK.FIDI.FK ...EEGSNLTSY 728
Thermolysin     142 HELTHAVTD YTAG.LIYQN E...SGAI.NE A..ISDI.FG TLVEFYAN.... 181
Listeria mpl    388 HELTHAVIQ YSAG.LEYEG Q...SGAL.NE S..FADV.FG ....YPIAPNHW 427
Elastase        140 HEVSHGFTE QNSG.LIYRG Q...SGGM.NE A..FSDM.AG EAADFYMR.... 179
Serratia npr    209 HEIGHAL.. ...G.LSHPG D..YNAGEGN. PTYN.DVTYA EDTRQFS.LMSY 249
Tetanus Toxin   233 HELIHVLHG LY.G.MQVSS HEIIPSKQ..E I.YMQHTYPI .SAEEL..... 272
Bot D           229 HELTHSLHQ LY.G.INIPS DKRIRPQ.VSE G.FFSQDGPN VQFEEL..... 270
Rat apN         387 HELAHQWFG NLVT.VDWWN DLWL.....NE G..FASYVEP LGADYAE.P.TW 428
                    88  843   3 37 5   3 3   4     6     5 5  3     53
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Fig. 1. Alignment of lethal factor with other metalloproteases. The common HEXXH motif is in bold. Listeria mpl, *Listeria monocytogenes* metalloprotease (Domann *et al.*, 1991); elastase, *Pseudomonas aeruginosa* elastase (Bever and Iglewski, 1988); Serratia npr, *Serratia marcescens* neutral protease (Braunagel and Benedik, 1990); BotD, *Clostridium botulinum* neurotoxin type D (Binz *et al.*, 1990); Rat apN, Rat aminopeptidase N (Watt and Yip, 1989). LF residues which were mutated in this study are marked with an asterisk. Residues which are identical to LF residues in two or more other proteases are indicated by a number, 3 through to 8, which represents the frequency of the LF residue at that position. The initial alignment was performed with the GCG program PILEUP and then refined by hand.

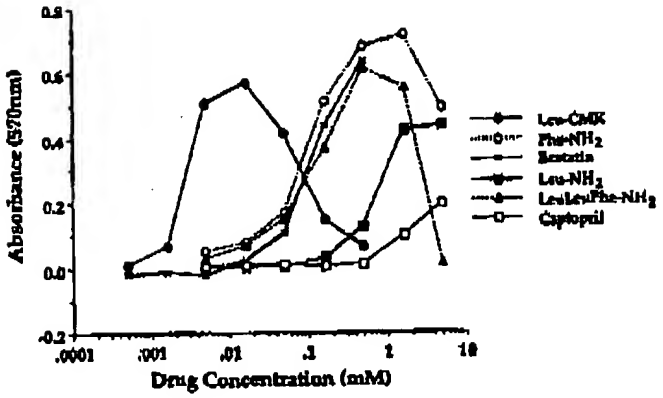

Fig. 2. Drug protection of J774A.1 cells from lethal toxin. J774A.1 cells were pre-incubated with drugs for 30–60 min prior to the addition of lethal toxin. Four hours after addition of toxin, MTT was added to a final concentration of 0.4 mg ml^{-1}. After an additional 30 min incubation the media was aspirated and the dye was solubilized and the colour quantified at 570 nm. Cells treated with toxin only were killed and did not produce any colour (data not shown). Each sample was tested in triplicate and subsequent experiments with the same drugs at equivalent concentrations had similar results.

originally isolated from cultures of *Streptomyces olivoreticuli* and shown to be an inhibitor of leucine aminopeptidase and aminopeptidases B and M (Umezawa *et al.*, 1976), was very effective at blocking LT activity (Fig. 2). Other Leu and Phe derivatives protected cells, with the Phe peptides (Phe-NH_2, LeuLeuPhe-NH_2) generally being more potent than the corresponding Leu (Leu-NH_2) peptides (Fig. 2). Captopril, a potent inhibitor of the angiotensin-converting enzyme, which also inhibits tetanus toxin and botulinum A and B neurotoxins *in vivo*, was only slightly inhibitory at 10 mM (Fig. 2) (Schiavo *et al.*, 1992a). Phosphoramidon, a strong inhibitor of thermolysin and neutral endopeptidases, had no effect on LT activity (data not shown).

Assay of LF for proteolytic activity

LF was examined for its ability to cleave several common protease substrates. Casein, albumin, and gelatin were used as substrates to test the general protease activity of LF. No release of small peptides or amino acids was detected. Similarly when leucine, lysine–alanine and glycine–phenylalanine beta-naphthylamides were incubated with LF to assay aminopeptidase activity, none was observed (data not shown).

We previously reported that incubation of intrinsically labeled ([^{3}H]-leucine or [^{35}S]-methionine) protein extracted from J774A.1 cells with LF resulted in the release of radioactivity into the assay buffer (Klimpel *et al.*, 1993). Subsequently we constructed a *B. anthracis* transposon mutant that lacks the ability to produce an extracellular protease (K. R. Klimpel, J. M. Hornung, S. Haley, and S. H. Leppla, unpublished). Incubation of intrinsically labeled protein extracted from J774A.1 cells with LF purified from this strain did not result in the release of any radioactive peptides into the buffer. We conclude that the activity reported was due to contamination of our LF by trace amounts of the extracellular protease produced by *B. anthracis*. In view of the requirement of EF for calmodulin as an enzymatic cofactor, we also tested whether proteolysis was enhanced by an extract of macrophages. We found no evidence for cleavage by LF of any of the substrates tested.

Zinc binding to LF

LF was tested for its ability to bind zinc in solution. After removal of metal ions from LF with EDTA, the protein was incubated with ^{65}Zn. Unbound ^{65}Zn was removed from the protein solution by concentration dialysis. LF treated in this manner bound considerable amounts of ^{65}Zn (Fig. 3). ^{65}Zn binding was optimal between pH 7.0–8.0 in the presence of 100 μM $CaCl_2$ (data not shown). Under these conditions each LF molecule bound about one zinc atom (calculation not shown). The bound ^{65}Zn

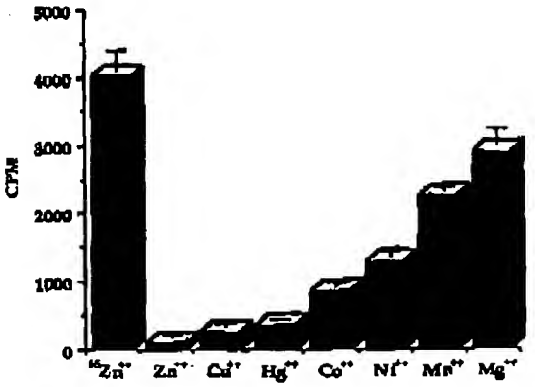

Fig. 3. ^{65}Zn binding to lethal factor. LF (30 μg) was incubated with ^{65}Zn (5 μM) in 100 μM $CaCl_2$, 10 mM Tris-HCl, pH 7.5, alone or in the presence of a 500-fold molar excess of other divalent cations for 1 h at 22°C. Unbound ^{65}Zn was removed by concentration dialysis with Centricon 50 concentrators (Amicon). Radioactivity associated with concentrated protein samples was determined in a gamma counting system with 13.04% efficiency for ^{65}Zn (Beckman). Error bars indicate the standard error of each data set.

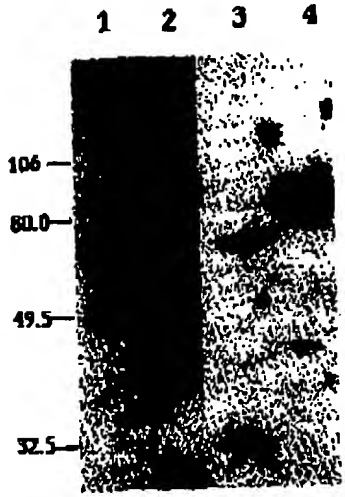

Fig. 4. ^{65}Zn binds to lethal factor immobilized on a PVDF membrane. Protein was separated by 10% SDS–PAGE. Proteins were stained with Coomassie brilliant blue or transferred to an Immobilon PVDF membrane. The membrane was incubated with ^{65}Zn, washed, dried and autoradiographed (see the *Experimental procedures*). Lane 1, PA (5 μg); lane 2, PALF (5 μg); lane 3, PA (5 μg); lane 4, PALF (5 μg). The protein in lanes 1 and 2 were visualized after staining with Coomassie brilliant blue R-250, while lanes 3 and 4 were visualized by autoradiography after the membrane was exposed to X-ray film for 72 h at 22°C.

could be displaced from LF by incubation in the presence of several divalent metal ions (Fig. 3). A 500-molar excess of zinc, copper or mercury prevented LF from binding ^{65}Zn. Nickel and cobalt displaced more than half of the bound ^{65}Zn while magnesium and manganese displaced only small amounts. These results are very similar to those found for tetanus toxin (Wright *et al.*, 1992).

Zinc binding was also measured by a zinc-blotting method (see the *Experimental procedures* and Wright *et al.*, 1992). Equal amounts of PA and LF were electrophoresed through a 10% SDS–polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. The membrane was washed twice with 5 mM EDTA and then rinsed with metal-free water. The filter was incubated with ^{65}Zn, washed, dried and autoradiographed. LF bound ^{65}Zn while no significant binding was observed for PA (Fig. 4, lanes 3 and 4). Optimal binding was achieved in the presence of 100 μM $CaCl_2$ at pH 7.5.

Residues at the HEXXH motif are essential for maximal zinc binding and for LF activity

The crystal structures of thermolysin, *Pseudomonas aeruginosa* elastase, and *B. cereus* neutral protease have been determined (Matthews *et al.*, 1974; Thayer *et al.*, 1991; Pauptit *et al.*, 1988). In addition to the identification of the HEXXH motif in LF, these crystal structures provided the framework from which we determined which residues in LF may be critical for activity. LF residues H-686, E-687 and H-690 are analogous to thermolysin residues H-142, E-143 and H-146, respectively. In thermolysin, H-142, H-146 and E-166 bind zinc (Matthews *et al.*, 1974). LF has no glutamic acid residue analogous to E-166. The nearest glutamic acid residues in LF are E-720 and E-721.

The recombinant polymerase chain reaction (PCR) (Horton *et al.*, 1990; Johnson *et al.*, 1993) was used to make specific substitutions in the LF gene. Residues H-686, H-690, E-720, and E-721 were substituted with A, and residue E-687 was substituted with C. Following PCR, each mutant construct was sequenced to confirm that only specified mutations were introduced. LF mutant proteins were made as fusions to the PA protein and expressed in *B. anthracis* as will be described elsewhere. The fusion proteins were cleaved with factor Xa or trypsin and the LF or mutant LF proteins were purified.

The amount of ^{65}Zn bound by each mutant LF was determined in solution. LF mutants 1, 2 and 3 bound less ^{65}Zn than did LF (Fig. 5). LF 1 (A^{686}) bound about 85% of wild type, while LF 2 (A^{690}) bound only 45%. The double mutants, LF 3 ($A^{686}A^{690}$), bound only about 13%, less than either of the single mutants. The amount of radioactive zinc bound by LF 4 (C^{687}) and LF 5 ($E^{720,721}$) was not significantly different from the amount bound by LF ($P = 0.28$ and $P = 0.68$, respectively).

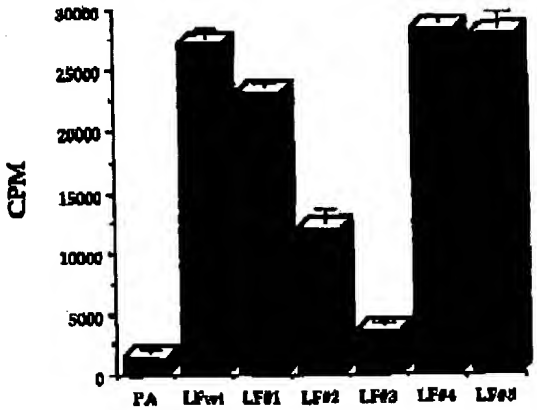

Fig. 5. ^{65}Zn binds to lethal factor and lethal factor mutants in solution. Two-hundred micrograms of PA, LF or LF mutant protein were incubated with ^{65}Zn (6 μM) in 100 μM $CaCl_2$, 10 mM Tris, pH 7.5 for 1 h at 22°C. Unbound ^{65}Zn was removed by two cycles of concentration dialysis with Centricon 50 concentrators (Amicon). Radioactivity of the concentrated protein samples was determined in a gamma counting system with 13.04% efficiency for ^{65}Zn (Beckman). Error bars indicate the standard error of each data set.

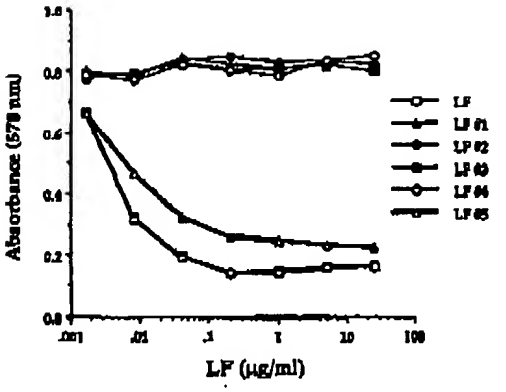

Fig. 6. Toxicity of mutated LF proteins. Mutated LF proteins were assayed for functional activity with RAW264.7 cells in the presence of PA (250 ng ml^{-1}). Cell viability was determined using MTT as described previously (Quinn *et al.*, 1991). Native and mutated LF proteins were expressed as PA protein fusions, cleaved with factor Xa and purified for use in this assay.

The activity of the purified LF proteins was determined by their ability to kill RAW264.7 cells in culture (Fig. 6). Native LF and the double mutant LF 5 ($A^{720,721}$), killed cells in a dose-dependent manner (native LF, EC_{50} of 6 ng ml^{-1}; LF 5 ($A^{720,721}$), EC_{50} of 11 ng ml^{-1}) while mutants LF 1 (A^{686}), LF 4 (C^{687}), LF 2 (A^{690}) and the double mutant LF 3 ($A^{686,690}$) failed to show any toxicity at concentrations up to 20 μg ml^{-1}.

These results are in general agreement with the zinc-binding data. The mutant proteins that bound substantially less zinc than the native protein (LF 1–3) were not toxic. Because the co-ordination of zinc depends on interactions with several amino acids the decreased ability of the double mutant to bind zinc, when compared to the single mutants, was anticipated. If E^{720} or E^{721} were involved in co-ordinating zinc as we predicted, we would have expected that their replacement in LF 5 would have had an effect on the protein's ability to bind zinc and intoxicate cells. LF, like the *Serratia marcescens* neutral protease and the *P. aeruginosa* alkaline protease, may have the third zinc ligand on the amino-terminal side of the consensus zinc metalloprotease site (Braunagel and Benedik, 1990; Okuda *et al.*, 1990). Determination of the third zinc ligand may be achieved by more exhaustive mutational analysis or by solving the crystal structure of LF.

Taken together, these data provide substantial evidence that LF is a zinc metalloprotease. This possibility would be confirmed by identification of an actual substrate. Like tetanus toxin and the botulinum neurotoxins, LF probably has a very narrow substrate specificity, perhaps a single protein or protein family (Schlavo *et al.*, 1992a; 1993; McMahon *et al.*, 1993). Identification of a substrate may prove to be difficult. Comparison of macrophage cell lines that are sensitive to the toxin with resistant lines may help distinguish potential LF targets.

Experimental procedures

Reagents and general procedures

Restriction endonucleases and DNA-modifying enzymes were purchased from GIBCO/BRL, Boehringer Mannheim, or New England Biolabs. Oligonucleotides were synthesized on a PCR Mate (Applied Biosystems) and purified with oligonucleotide purification cartridges (Applied Biosystems). DNA sequencing to confirm constructs was performed with the Sequenase Version 2.0 sequencing kit from US Biochemical Corp. according to the manufacturer's instructions. DNA fragments were purified after electrophoresis from agarose gels with a QIAEX gel extraction kit (Qiagen). Protein concentrations were determined with the BCA reagent (Pierce) using bovine serum albumin (BSA) as a standard.

Zinc binding in solution

Purified proteins used for zinc binding in solution were first passed through a Sephadex G-25 column (PD-10, Pharmacia) that had been equilibrated with 10 mM Tris, pH 7.5, 100 μM $CaCl_2$. ^{65}Zinc (2.07 mCi mg^{-1}, Du Pont-NEN) was added to the protein solution to a final concentration of 5 μM, final volume 2 ml, and incubated at 22°C for 1 h. Unbound zinc was removed by two cycles of concentration dialysis with Centricon 50 concentrators (Amicon).

Zinc blot

After PA and PALF fusion protein were treated with small amounts of trypsin (0.5 μg ml^{-1} for 30 min at 23°C) to cleave the 20 kDa amino-terminal fragment, the proteins were separated by 10% SDS–PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corp.) previously wetted with methanol. Membranes were washed twice in 5 mM EDTA, 10 mM Tris, pH 7.5, washed five times in water, and then incubated for 3 h in 5 μM $^{65}ZnCl_2$, 100 μM $CaCl_2$, 10 mM Tris, pH 7.5. Membranes were washed three times in 100 μM $CaCl_2$, 10 mM Tris, pH 7.5 to remove unbound ^{65}Zn, and exposed to X-ray film. All reagents were pretreated with Chelex-100 resin (Bio-Rad) to remove metal ions.

Cytotoxicity measurements

Viability of toxin-treated macrophages was measured as described previously (Quinn *et al.*, 1991), using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Briefly, RAW264.7 or J774A.1 macrophages were grown to 50% confluence in 96-well plates, then PA (250 ng ml^{-1}) and varying amounts of LF, LF plus inhibitor, or inhibitor alone were added, and incubation was continued for 4 h. MTT was added to 0.5 mg ml^{-1}, and after 45–60 min, the medium was aspirated and the blue pigment dissolved and the absorbance measured in a multiwell plate reader.

Site-directed mutagenesis of LF

Recombinant overlapping PCR was used to replace LF residues His-686, His-690, Glu-720 and Glu-721 with Ala, and to replace Glu-687 with Cys. Briefly, two complementary mutagenic primers spanning the site at which the mutation is to be placed are used in the first round of PCR to amplify two segments of a gene or gene fragment. This is followed by a second recombinant PCR, using the purified products of the first round, which reconstitutes the gene or gene fragment containing the site specific mutation(s). For a detailed description of this procedure see Horton *et al.* (1990). One primer used in the second recombinant PCR reaction introduced a unique *Bam*HI site beyond the 3′ end of the coding sequences for the LF gene. The products from the recombinant round of PCR were purified after agarose gel electrophoresis and digested with restriction enzymes *Xho*I and *Bam*HI. The large *Xho*I–*Bam*HI fragment from plasmid pPA^{1-164}–Xa–LF was purified by agarose gel electrophoresis and ligated to each of the mutated LF *Xho*I–*Bam*HI fragments described above. DNA sequences in the mutated regions were confirmed by sequencing each clone used to transform *B. anthracis*. All five plasmids were introduced into *B. anthracis* UM23C1-1 by electroporation (Quinn and Dancer, 1990). Plasmid pPA^{1-164}–Xa–LF is an expression plasmid which codes for the high-level expression in *B. anthracis* of LF fused to the PA residues 1–164 and will be described in detail elsewhere.

Production and purification of PA^{1-164}–Xa–LF fusion proteins from B. anthracis

PA proteins and PA^{1-164}–Xa–LF fusion proteins were produced by growth of the transformed *B. anthracis* strains in FA medium as previously described for PA (Klimpel *et al.*, 1992). Cultures (5 l) were grown 14–18 h at 37°C in a 10 l Bio-FloIV fermentor (New Brunswick Scientific), aerated at 3 l air per min, with stirring at 450 r.p.m. After growth, the culture was cooled to 12–15°C, EDTA added to 1 mM, and the pH adjusted to 8.0. Bacteria were removed by centrifugation at 5020 × *g* at 4°C for 30 min. Proteins were precipitated by addition of 0.47 g ammonium sulphate per ml of supernatant. The precipitated proteins were concentrated by centrifugation, dialysed, and purified by chromatography on hydroxyapatite (Calbiochem) and MonoQ columns (Pharmacia) (Quinn *et al.*, 1988; Leppla, 1991a). Pa^{1-164}–Xa–LF fusions eluted from MonoQ resin at 300–320 mM NaCl in pH 7.5 buffers. The purified fusion proteins were diluted to 1.0 mg ml^{-1} with cleavage buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM $CaCl_2$, pH 8.0) and incubated with factor Xa (20 μg ml^{-1}) for 12 h at 23°C. The resulting LF was purified by anion exchange chromatography on a MonoQ column.

Acknowledgements

The authors are indebted to Ray Fields and Sholla Haley for technical assistance. We would like to acknowledge Peter FitzGerald for his assistance with protein sequence analysis. We thank Jerry M. Keith for the support and encouragement that made this work possible.

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